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THE ANAEROBIC BIODEGRADATION OF POLY(3-HYDROXYALKANOATES)

BY

KAREN BUDWILL ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

IN

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA
SPRING, 1995

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To my parents

For all their love, support and encouragement over the years

ABSTRACT

The biodegradation of poly(3-hydroxyalkanoates) (PHAs) under different anaerobic conditions was examined using the techniques of anaerobic microbiology and analytical chemistry. Methanogenic environments sampled included sewage sludge, pond sediment and rumen fluid. Nitrate-reducing consortia were established using activated sludge. Attempts were made to establish ferric iron- and sulfate-reducing consortia from sewage sludge and spring water, yet it could not be demonstrated that the mixed cultures were capable of degrading PHAs.

PHAs were shown to be biodegradable in activated sludge under nitrate-reducing conditions. A positive correlation between carbon dioxide production and nitrate reduction was demonstrated. Nitrous oxide accumulated as the main N-containing end product of nitrate reduction. The amount of PHAs in activated sludge cultures decreased approximately 20% within 40 days of incubation.

PHAs were fermented to methane and carbon dioxide within 16 days by an anaerobic sewage sludge consortium. The cultures adapted quickly to metabolize the PHAs, and between 83 and 96% of the substrate carbon was transformed to methane and carbon dioxide. PHAs were fermented to methane and carbon dioxide after 10 weeks by a sediment consortium as well, however, only 43 and 57% of the substrate carbon was transformed to methane.

Although it could not be demonstrated that PHAs were biodegraded by a rumen fluid consortium, a facultative anaerobic bacterium, identified as a *Staphylococcus* sp., that could grow on PHAs was isolated from rumen fluid. A depolymerase was secreted into the culture fluid during both the exponential and stationary growth phase when PHA granules served as carbon substrate. The majority of the depolymerase activity was not detected in the culture supernatant but was found to be associated with the granules.

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LIST OF ABBREVIATIONS

3HB	3-hydroxybutyrate
3HD	3-hydroxydecanoate
3HH	3-hydroxyheptanoate
3HO	3-hydroxyoctanoate
3HV	3-hydroxyvalerate
ATCC	American Type Culture collection
BESA	Bromoethane sulfonic acid
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung Von Mikroorganismen
GC	Gas chromatography
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HO)	Poly(3-hydroxyoctanoate)
P(3HV)	Poly(3-hydroxyvalerate)
PHA	Poly(3-hydroxyalkanoate)
RNA	Ribonucleic acid
SEM	Scanning Electron Microscopy
SRB	Sulfate-reducing bacteria
TCA	Tricarboxylic acid cycle
UV	Ultraviolet

1. INTRODUCTION AND LITERATURE REVIEW

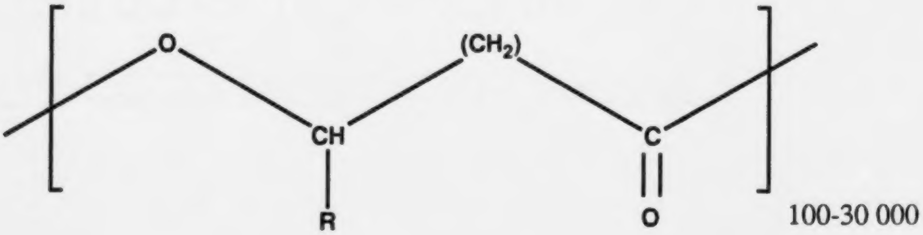
Because petroleum based plastics are non biodegradable, they form a very visible type of solid waste pollution. During the mid 1980's to early 1990's, the general public and legislation demanded a reduction in plastic waste. Recycling of plastics has been touted as a means of reducing waste but it has not reached its full potential and the process is still more expensive than manufacturing virgin polymer. As a consequence, the plastics industry began to develop natural polymers such as starch blends, polylactic acid and bacterial poly(3-hydroxyalkanoates) or PHAs, as biodegradable plastics. PHAs have been singled out as excellent candidates for replacing conventional plastics in a number of applications (Holmes, 1985; Logsdon, 1989) and has become the focus of numerous studies. Recent, excellent reviews on PHAs have been written by Anderson and Dawes (1990), Doi (1990) and Steinbüchel (1991).

1.1 PHA Characteristics

1.1.1 Structure and Physical Properties of PHAs

PHAs are microbially produced, water insoluble, optically active polyesters that are comprised of alkanoic acids containing a hydroxyl group as at least one functional group in addition to the carboxy groups (Steinbüchel, 1991).

The general formula is as follows:



where R = *n*-alkyl pendant group of variable chain length.

The following functional groups have been detected:

R=hydrogen: 3-hydroxypropionate (3HP)
R=methyl: 3-hydroxybutyrate (3HB)
R=ethyl: 3-hydroxyvalerate (3HV)
R=*n*-propyl: 3-hydroxycaproate (3HC)
R=*n*-butyl: 3-hydroxyheptanoate (3HH)
R=*n*-pentyl: 3-hydroxyoctanoate (3HO)
R=*n*-hexyl: 3-hydroxynonanoate (3HN)
R=*n*-heptyl: 3-hydroxydecanoate (3HD)
R=*n*-octyl: 3-hydroxyundecanoate (3HUD)
(Steinbüchel, 1991; Brandl *et al.*, 1990).

Poly(3-hydroxybutyrate) P(3HB) was first described by Lemoigne in 1925 who isolated the polymer from *Bacillus megaterium* (Doi, 1990). It was not until some 60 years later that Wallen and Rohwedder (1974) extracted and characterized heteropolymers from activated sludge. Findlay and White (1983) detected 11 short chain 3-hydroxyacids, the principal ones being 3HB and 3HV, in polymers extracted from marine sediment. PHAs other than P(3HB) synthesized by defined bacterial cultures were only described after 1982 (Steinbüchel, 1990).

P(3HB) is a compact right-handed helix with a 2-fold screw axis fiber repeat of 0.596 nm (Cornibert and Marchessault, 1972). Poly(3-hydroxyvalerate) P(3HV) also has a 2-fold helical conformation and a fibre repeat of 0.556 nm (Doi, 1990). Polymer strands are crosslinked to adjacent strands by hydrogen bonds formed between water molecules and carbonyl groups of the polyester backbones (Lauzier *et al.*, 1992). The polyesters are believed to exist in only [R]-configuration (D(-) in traditional nomenclature) in the chiral center of 3HB acid. In polymer chemistry terms, PHAs are said to be perfectly isotactic in that the side groups are all on one side of the polymer chain when the chain is fully extended. Solvent-extracted polyesters from different bacteria have molecular weights of up to 2×10^6 (Brandl *et al.*, 1990).

The physical properties of P(3HB) have often been compared to that of polypropylene and are summarized in Table 1.1. The chemical properties are completely different as P(3HB) possesses far inferior solvent resistance but better natural resistance to UV weathering than polypropylene. Physically, P(3HB) is stiffer and more brittle than polypropylene (Holmes, 1985). But physical and mechanical properties can change considerably as a function of monomer composition, for example, copolymers of 30 to 40 mol % 3HV units have a reduced melting temperature of approximately 75°C (from 180°C for P(3HB)) and are more flexible and tougher than P(3HB) (Brandl *et al., 1990*).

Table 1.1 Comparison of physical properties of polypropylene (PP) and P(3HB) (Brandl *et al.*, 1990).

Parameter	PP	P(3HB)
Melting Point T_m (°C)	171-186	171-182
Glass Transition Temperature T_g(°C)	-15	5-10
Crystallinity (%)	65-70	65-80
Density (g/cm^3)	0.905-0.94	1.23-1.25
Molecular Weight Mw (x10^5)	2.2-7	1-8
Polydispersity Index	5-12	2.2-3
Tensile Strength (MPa)	39	40
Extension to Break (%)	400	6-8

1.1.2 Distribution and Accumulation of PHAs

PHA accumulation occurs in a wide variety of taxonomically different groups of prokaryotes. Most of these prokaryotes are capable of accumulating PHAs from 30 to 80 percent of their cellular dry weight (Brandl *et al.*, 1990). PHAs have been detected in both Gram negative and positive bacteria, aerobic and in many anaerobic bacteria such as clostridia (Brandl *et al.*, 1990) and *Syntrophomonas wolfei* (McInerney *et al.*, 1992) (Steinbüchel, 1991). Most non-sulfur and sulfur purple bacteria produce PHA (Liebergesell *et al.*, 1991). The green gliding bacteria, *Chloroflexus auranthiacus* can accumulate small amounts of PHA. PHA has been detected in aerobic photosynthetic bacteria such as cyanobacteria as well as in enterobacteria such as *Escherichia coli*, although PHA has only been found in the membrane of this microorganism (Reusch, 1992). The genes for the P(3HB) biosynthetic pathway from *Alcaligenes eutrophus*, however, were cloned and expressed in *E. coli* (Slater *et al.*, 1988; Schubert *et al.*, 1988).

Staphylococcus aureus, Staphylococcus xylosus, and *Staphylococcus epidermidis* (Szeweczyk and Mickucki, 1989) and some archaebacteria such as halobacteria (Rodriguez-Valera and Lillo, 1992) are also capable of synthesizing PHA. Recently the biosynthetic genes from *A. eutrophus* were cloned into *Arabidopsis thaliana* plants and expressed (Poirier *et al.*, 1992a; Poirier *et al.*, 1992b). P(3HB) produced by the plants was stored in inclusion bodies which were similar in size and appearance to P(3HB) inclusion bodies accumulated in bacteria.

PHA accumulation has not been detected in methanogenic bacteria, lactic acid bacteria and green sulfur bacteria (Steinbüchel, 1991). It has yet to be determined whether these bacteria are impaired in the synthesis of these polymers for physiological, biochemical, genetic or evolutionary reasons.

The most common form of PHA produced by microorganisms is P(3HB). However, by varying, the substrates during growth of some bacteria, the production of PHAs of varying composition can be controlled. A glucose-utilizing mutant of *A. eutrophus* can produce up to 80% (w/w) P(3HB) when glucose serves as the carbon source. When alkanoic acids with odd carbon numbers such as propionic and pentanoic acid are given to the cultures of *A. eutrophus* a random copolymer containing both 3HB and 3HV monomer units, P(3HB-co-3HV), is produced (Holmes, 1985). The 3HV content is dependent on the ratio of odd carbon number alkanoic acid to glucose in the medium during the polymer accumulation stage. *A. eutrophus* is also capable of accumulating 3HP, 4HB and 5HV monomers as part of the polymer.

Pseudomonas oleovorans ATCC 29347 when grown on *n*-octane (given at a concentration of 50% v/v) as sole carbon and energy source, produces a polymer comprised of 3HO as the major constituent (De Smet *et al.*, 1983). Haywood *et al.* (1989) screened various other *Pseudomonas* species for growth and PHA accumulation with C_4 to C_{10} straight chain alkanes, alcohols and alkanoic acids as the sole carbon source. The range of substrates that supported PHA synthesis was different and also differed from that in *P. oleovorans*. However, the pattern of PHA accumulation was related to that in *P. oleovorans* since 3-hydroxyacids possessing the same carbon chain length as the substrate, or differing by multiples of 2-carbon units were the major monomer units found in the polyesters and no 3HB monomer which is characteristic of P(3HB) was present in the polymer (Steinbüchel, 1991).

Other bacteria reported to produce PHAs include Gram positive bacteria such as *Rhodococcus* sp. which can synthesize P(3HB-co-3HV) from carbohydrates such as fructose or glucose or from organic acids such as succinate, acetate or lactate (Steinbüchel, 1991). *Azotobacter vinelandii* strain UWD (ATCC 53799) can accumulate P(3HB) on complex substrates such as beet molasses (Page, 1989; 1992). *A. vinelandii* strain UWD can also produce P(3HB-co-3HV) by the controlled feeding of valerate during the polymer accumulation stage (Page *et al.*, 1992).

1.1.3 Functions of PHAs

In general, environmental conditions and the physiological abilities of the bacteria control the quantitative formation of the storage polymer. Low concentration or total absence of a variety of different nutrients can induce or stimulate the formation of PHA. PHA acts as a source of energy and carbon for bacteria under conditions of starvation. The synthesis of these high molecular weight polymers allow bacteria to store large quantities of reduced carbon without affecting the osmotic pressure of the cell since PHAs are chemically and osmotically inert within the cell (Brandl *et al.*, 1990). By internally storing the polymer, the carbon source is unavailable for competing organisms (Steinbüchel, 1991).

The presence of PHA aids in the survival of some bacteria under conditions of environmental stress other than starvation, such as extremes in osmotic pressure, desiccation or UV irradiation (Tal and Okon, 1985). PHA serves as a carbon and energy source for spore formation and encystment in *Bacillus* and *Azotobacter* species respectively (Brandl *et al.*, 1990). PHA also acts as an oxidizable substrate for azotobacters when no appropriate exogenous substrate is available in order to provide respiratory protection to its nitrogenase enzyme (Senior and Dawes, 1971). Since P(3HB) was found in prokaryotic and eukaryotic membranes, it has been postulated that it plays a role in the regulation of intracellular calcium concentrations and in calcium signaling as well as in DNA uptake (Reusch, 1992).

1.1.4 Inclusion Bodies

PHAs are formed within the cytoplasm of the cell as granular inclusions that range from 0.2 to 0.5 μm diameter (Steinbüchel, 1991). There are usually several inclusion bodies present per cell, though the number varies from bacterium to bacterium.

Approximately 2% (w/w) protein and 0.5% (w/w) lipid have been found to be associated with the inclusion bodies (Lusty and Doudoroff, 1966). It is assumed the lipids form a monolayer membrane on the surface of the inclusion body and the proteins are involved in the synthesis and depolymerization of PHA (Anderson and Dawes, 1990).

The native structure of the inclusion bodies and the physical state of PHA within it is a matter of controversy. It was believed PHA was in a crystalline state within the inclusion bodies, but recent evidence from ^{13}C-NMR spectroscopy and X-ray diffraction studies indicate that at least 70% of the polyester in the native inclusion bodies occurs as a very mobile amorphous elastomer (Bernard and Sanders, 1989). Approximately 5-10% (w/w) water has been found associated with an inclusion body (Lauzier *et al.*, 1992). Therefore, water may be an integral component of the inclusion bodies and may act as a plasticizer for PHA (Steinbüchel, 1991). It has been proposed that PHA synthase and depolymerase are operable in the mobile hydrated polymers.

Bonthrone *et al.* (1992) suggested that cells are able to prevent PHA crystallization *in vivo* simply because the crystallization kinetics are so slow (several months). When inclusion bodies are disrupted by centrifugation, they readily coalesce into larger masses and single nucleation events will crystallize a much larger amount of material than in an individual inclusion body and lead to an increase in the apparent crystallization rate (Bonthrone *et al.*, 1992; Lauzier *et al.*, 1992).

1.1.5 Detection, Isolation and Extraction of PHAs

PHAs can be observed under the light microscope as refractile bodies (Dawes and Senior, 1973) or else stained by Sudan Black B or the more specific Nile Blue A dye which gives a bright orange fluorescence at a wavelength of 460 nm (Anderson and Dawes, 1990).

The isolation of native polymer inclusion bodies was achieved by repeated centrifugation of DNase treated cell extracts layered on glycerol (Merrick and Doudoroff, 1964) or by differential and density gradient centrifugation with glycerol (Griebel *et al.*, 1968). Inclusion bodies from *Zoogloea ramigera* were isolated by sucrose density gradient centrifugation after sonically disrupting the cells and collecting the extracts by centrifugation (Fukui *et al.*, 1976).

Organic solvents, mainly chlorinated hydrocarbons, readily extract PHAs from microorganisms (Anderson and Dawes, 1990; Doi, 1990). Alkaline sodium hypochlorite treatment will gradually digest cells thereby liberating PHA inclusion bodies (Williamson and Wilkinson, 1958). In both methods, the isolated PHA granules can be purified by washing with ethanol or methanol to remove lipids. Long incubations of cells in sodium hypochlorite often results in degradation of P(3HB) chains. To minimize degradation and the subsequent decrease in molecular weight, Berger *et al.* (1989) optimized biomass concentration, digestion time and pH of the hypochlorite solution. As well, Ramsay *et al.* (1990) included a surfactant pretreatment step to the alkaline treatment method to obtain PHA granules of high purity and molecular weight.

The above mentioned extraction techniques work well at the laboratory bench scale but become impractical at large scale fermentation levels. Not only is it expensive to use solvents but large quantities of chlorinated wastes are generated that need to be disposed. A process developed by Imperial Chemical Industries (ICI) in England used heat disruption, enzymatic digestions and peroxide treatment to extract PHA (Holmes and Lim, 1990; Marchessault *et al.*, 1990). This was followed by agglomeration of spray dried isolated PHA granules. Enzymatic treatment of the bacterial cells to solubilize all of the cell components except for PHA has been investigated by Griebel *et al.* (1968) and Harrison *et al.* (1991). Liberated PHA can then be separated from cell debris by centrifugation.

1.1.6 Commercial Applications of PHAs

The thermoplastic properties of P(3HB) were recognized in the early 1960's, however, it was not until the discovery in the 1980's, of P(3HB-co-3HV) with its improved physical and mechanical properties, that commercial application of PHAs occurred. ICI began fermenting *A. eutrophus* H16 on a large scale to produce P(3HB-co-3HV) under the trade name Biopol. The main application of Biopol was in packaging. It was used for films, coating on paper and blow molded bottles (Luzier, 1992). Shampoo bottles made entirely of Biopol were first test marketed in Germany in 1990 (Anderson and Dawes, 1990). Subsequently, several hair care companies in Japan also began producing bottles from Biopol (Rogers, 1992). PHAs could also be used in personal hygiene products, food wrapping and garbage bags and other items that are readily disposed (Luzier, 1992) as well as agricultural mulching films (Doi, 1990). Since PHAs are optically active, the monomers of PHAs have been considered as a source for the synthesis of enantiomeric pure chemicals (Steinbüchel, 1991).

PHAs are non toxic, biocompatible and slowly hydrolyzed in the body and therefore lend themselves to several medical applications such as surgical sutures and swabs, wound dressings and lubricants for surgeons' gloves (Holmes, 1985). As well, PHAs can be used to coat medications and applied as slow release drugs (Saito *et al.*, 1991).

1.2 Intracellular Metabolism

1.2.1 Biosynthesis of PHAs

Three different basic biosynthetic pathways for PHA are known to exist. The *A. eutrophus* pathway, so named because the pathway has been most extensively studied in this organism (Steinbüchel, 1991), is composed of three steps (Figure 1.1) in which PHB is synthesized from acetyl coenzyme A (AcCoA). First, the enzyme ß-ketothiolase catalyzes the condensation of two AcCoA moieties. In the second step, an NADPH-dependent acetoacetyl-CoA reductase catalyzes the stereo selective reduction of acetoacetyl-CoA (AcAcCoA) formed in the first reaction to D(-)-3-hydroxybutyryl CoA. The third reaction involves the linkage of D(-)3-hydroxybutyryl moiety to an existing polyester molecule by an ester bond. This reaction is catalyzed by the enzyme P(3HB) synthase. The pathway can be summarized by the following equation:

$$2\ \text{AcAcCoA} + \text{NADPH} + \text{H}^{+} + \text{P(3HB)}_{n} \rightarrow \text{NADP}^{+} + 2\ \text{CoASH} + \text{P(3HB)}_{n+1}$$

where P(3HB)_n and P(3HB)_{n+1} represent polymer molecules of n plus n+1 monomer units respectively (Collins, 1987). The hydrolysis of the CoA ester links provides the energy required for the condensation and polymerization of P(3HB).

The P(3HB) biosynthetic pathway in *A. vinelandii* strain UWD appears to be a typical *A. eutrophus* type pathway (Manchak and Page, 1994). An exception to this is the formation of P(3HB-co-3HV) by the strain. The 3HV subunits are generated by the ß-oxidation of odd number *n*-alkanoates and not by the condensation of propionyl-CoA and AcCoA (Page *et al.*, 1992).

The second pathway, the *Rhodospirillum rubrum* PHA biosynthetic pathway, consists of five steps and is only known to occur in this bacterium (Moskowitz and Merrick, 1969). Two stereo-specific enoylCoA hydratases are also involved. They

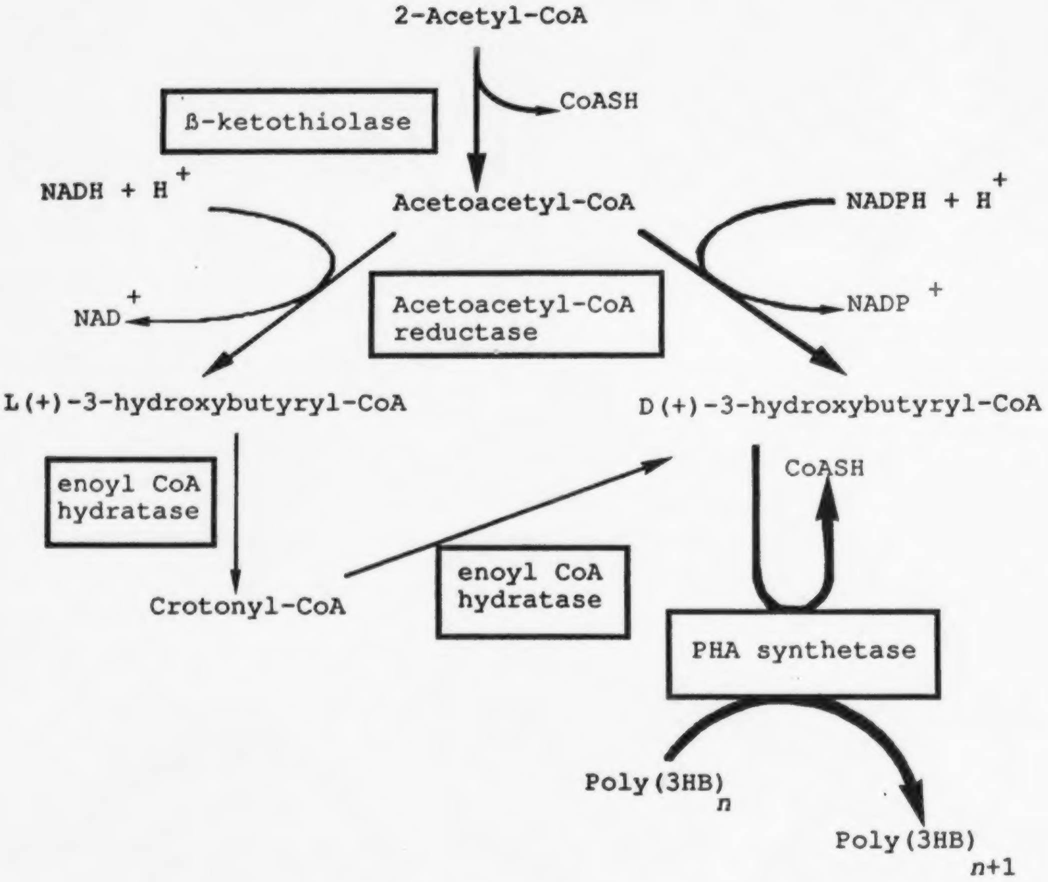

2-Acetyl-CoA
CoASH
ß-ketothiolase
NADH + H+
Acetoacetyl-CoA
NADPH + H+
NAD+
Acetoacetyl-CoA reductase
NADP+
L(+)-3-hydroxybutyryl-CoA
D(+)-3-hydroxybutyryl-CoA
enoyl CoA hydratase
CoASH
Crotonyl-CoA
enoyl CoA hydratase
PHA synthetase
Poly(3HB)n
Poly(3HB)n+1

Figure 1.1. *Alcaligenes eutrophus* (⟶) and *Rhodospirillum rubrum* (→) PHA biosynthetic pathway.

catalyze the conversion of L-(+)-3-hydroxybutyryl-CoA via crotonyl-CoA to D-(-)-3-hydroxybutyryl-CoA which is then polymerized to yield PHB as shown in Figure 1.1 (Moskowitz and Merrick, 1969).

The third pathway seems to be active in most pseudomonads belonging to the ribosomal RNA homology group I and is called the *Pseudomonas oleovorans* PHA biosynthetic pathway as it has been mostly studied in this organism (De Smet *et al.*, 1983; Brandl *et al.*, 1988). These pseudomonads accumulate PHA consisting of 3-hydroxy alkanoic acids of medium chain length if cells are cultivated on alkanes, alcohol or alkanoic acids. The intermediates of ß-oxidation which arise from the oxidation of activated fatty acids derived from the above substrates are likely directed to PHA biosynthesis in these bacteria (Figure 1.2) (Steinbüchel, 1991).

1.2.2 Intracellular Degradation of PHAs

The intracellular degradation or mobilization involves a depolymerase that is either associated with the PHA inclusion body or located in the cytoplasm depending on the microorganism (Anderson and Dawes, 1990). The resulting 3HB formed from P(3HB) degradation is converted to acetoacetate by ß-hydroxybutyrate dehydrogenase. It is then co-acylated by succinyl-CoA transferase to form AcAcCoA (Senior and Dawes, 1973).

The degradation of P(3HB) in *B. megaterium* requires a granule associated heat-labile factor, PHB depolymerase and an activator protein (Griebel *et al.*, 1968). *R. rubrum* was found to require a granule-associated, heat-labile factor, P(3HB) depolymerase, an oligomer hydrolase and a heat-stable activator protein for the intracellular degradation of P(3HB) (Griebel and Merrick, 1971).

1.2.3 Cellular Regulation of Synthesis and Degradation of P(3HB)

The key regulatory enzyme for PHA biosynthesis is 3-ketothiolase which is inhibited by high concentrations of free coenzyme A (CoA) (Jackson and Dawes, 1976; Oeding and Schlegel, 1973; Senior and Dawes, 1971). Under balanced growth conditions in the presence of excess carbon, AcCoA enters the TCA cycle for energy generation and formation of amino acids. This results in a high concentration of free CoA, and consequently P(3HB) synthesis is inhibited (Steinbüchel, 1991).

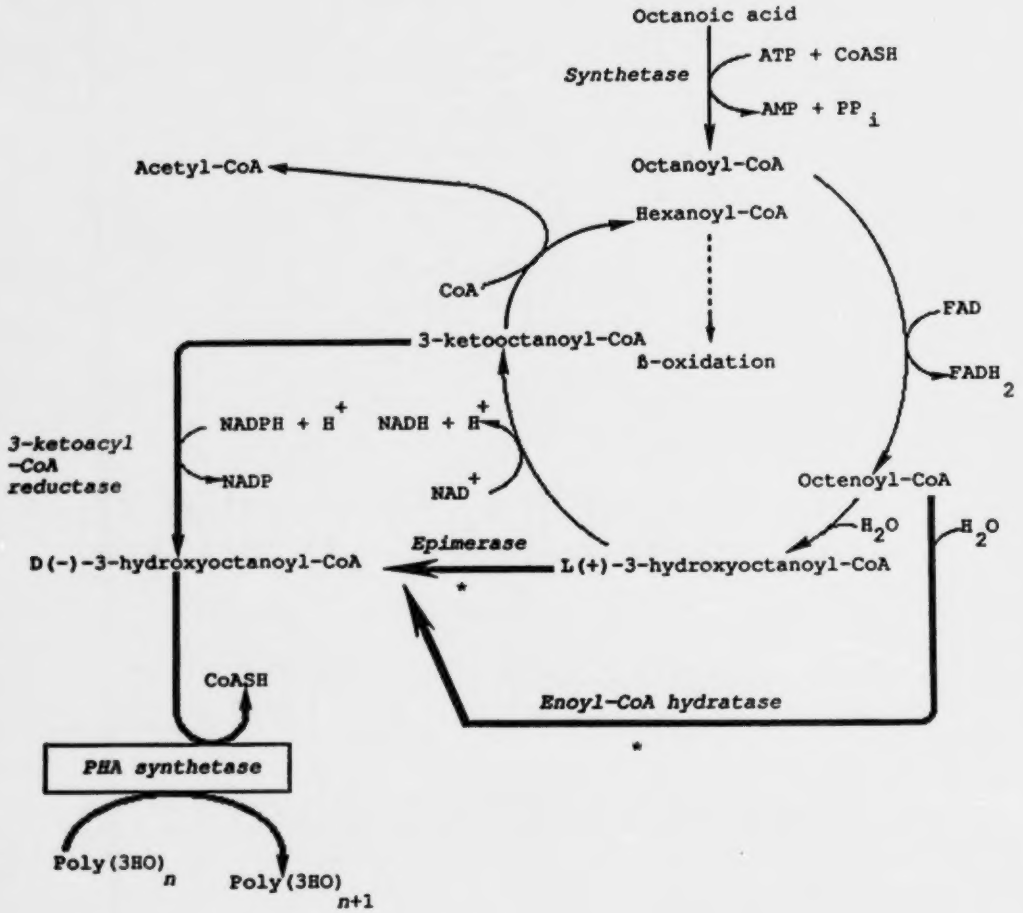

Octanoic acid
Synthetase
ATP + CoASH
AMP + PP_i
Octanoyl-CoA
Acetyl-CoA
Hexanoyl-CoA
CoA
FAD
$FADH_2$
3-ketooctanoyl-CoA
ß-oxidation
3-ketoacyl -CoA reductase
NADPH + H^+
NADP
NADH + H^+
NAD^+
Octenoyl-CoA
H_2O
H_2O
Epimerase
*
D(-)-3-hydroxyoctanoyl-CoA
L(+)-3-hydroxyoctanoyl-CoA
CoASH
Enoyl-CoA hydratase
*
PHA synthetase
$Poly(3HO)_n$
$Poly(3HO)_{n+1}$

Figure 1.2. *Pseudomonas oleovorans* PHA-biosynthetic pathway (⟶). *-speculation, has yet to be elucidated if these steps occur (Steinbüchel, 1991).

When an environmental stress, especially oxygen limitation, occurs in the presence of an excess carbon source, the NADH/NAD ratio increases (Anderson and Dawes, 1990). Citrate synthetase and isocitrate dehydrogenase are inhibited by NADH, and so AcCoA no longer enters the TCA cycle at the same rate as before the limitation (Doi, 1990). Instead, it is converted to AcAcCoA by 3-ketothiolase. Therefore, there is a greatly decreased flux of carbon through the TCA cycle under these conditions.

When the oxygen limitation is lifted, the CoA concentration increases because it is being released as AcCoA enters the TCA cycle. The high concentration of CoA inhibits 3-ketothiolase and consequently P(3HB) synthesis. P(3HB) degradation is controlled by the enzyme 3-hydroxybutyrate dehydrogenase which oxidizes the monomeric 3HB. This enzyme is subject to product inhibition by AcAc and NADH. P(3HB) synthesis is, therefore, linked with other metabolism in the cell and acts as a redox regulator within the cell.

Regulation of P(3HB) synthesis in *A. vinelandii* strain UWD is affected by a mutation leading to P(3HB) accumulation (Page and Knosp, 1989). The mutation is a defect in the respiratory oxidation of NADH and leads to increased levels of NADPH by the enzyme transhydrogenase (Manchak and Page, 1994). The increased concentration of NADPH causes feedback inhibition of citrate synthetase and isocitrate dehydrogenase which consequently slows the TCA cycle and increases the AcCoA to CoA ratio so that P(3HB) synthesis is favoured (Manchak and Page, 1994).

Experiments performed by Doi *et al.* (1990a) showed the cyclic nature of PHA synthesis and degradation. *A. eutrophus* cells were able to degrade P(3HB) and synthesize new polymer containing 3HV units under nitrogen limitation and in the presence of valeric acid. Likewise the copolymer was degraded in the presence of butyric acid. The regulation of polymer degradation is thus relaxed in these circumstances, while synthesis of a new polymer is simultaneously occurring.

1.3 PHA Biodegradation

1.3.1 Methods for Measuring PHA Biodegradation

A common method of determining whether a compound is being biodegraded by bacteria is to measure the concentration of the compound in the solution over a period of time. However, PHAs are insoluble in water and consequently following the decrease in substrate concentration as a measure of the extent of biodegradation is difficult. The most common method is to prepare solution cast films by dissolving a certain amount of PHA in chloroform and pouring the solution onto a glass surface. After the chloroform has evaporated and the resulting film allowed to age (Bloembergen *et al.*, 1986), strips can be cut from the film and exposed to test conditions. Loss of film weight and decrease in film thickness are measured as an indication of the degradation of PHA. Changes in molecular weight of PHAs can be measured by gel permeation chromatography high performance liquid chromatography (GPC-HPLC) (Doi *et al.*, 1990b).

In order to evaluate the mechanical properties of PHA films, standardized methods have been designed and outlined by the American Society for Testing Materials (ASTM), for example ASTM D882-91 and ASTM D638-91. Dog bone-shaped specimens are usually tested by applying forces in a single direction by an apparatus consisting of a machine with a pair of jaws which, during the test, are moved relative to each other, either together or apart, in a controlled manner (Nicholson, 1991).

The following are definitions of mechanical properties that can be tested (Morris, 1992) and are mentioned in discussions of particular studies in section 1.3.4. The tensile strength or stress is the maximum stretching that a material can withstand without breaking whereas stress at break refers to the amount of stress needed to fracture a polymer specimen. The degree to which a material can be stretched or distorted without breaking is called the extensibility of the polymer. The elongation to break, therefore, is the amount of stretching required to fracture a specimen with elongation defined as the increase in length produced in gage length of the test specimen by a tensile load. The elongation to break is often expressed as a percentage of its original size. Young's modulus is the ratio between tensile stress and elongation of a solid stressed in one direction.

1.3.2 Extracellular PHA Depolymerases

One of the first reports of P(3HB)-degrading microorganisms was by Chowdhury (1963) who isolated two pseudomonad strains from soil enrichment cultures that were capable of degrading this biopolymer. The extracellular depolymerase of one of the strains was investigated further and found to be composed of one homogeneous protein fraction. Among various substrates tested, only P(3HB) granules were able to induce the secretion of this depolymerase. Since then, further enrichments of PHA-degrading microorganisms from soils, air, marine and freshwater and anaerobic mud have yielded strains possessing similar and unique PHA depolymerases.

1.3.2.1 *Pseudomonas lemoignei*

Delafield *et al.* (1965b) isolated a number of aerobic pseudomonads from soil that were capable of using purified PHB as their sole carbon source. A new strain designated as *Pseudomonas lemoignei* was chosen for further studies. This Gram negative rod grew on acetate, butyrate, valerate, pyruvate, succinate, DL-3HB, the dimeric ester of D-3HB and P(3HB). It grew poorly, if at all, with propionate, L-malate, α-ketoglutarate and citrate (Delafield *et al.*, 1965b).

The P(3HB) depolymerase of *P. lemoignei* was produced constitutively in succinate- and acetate-containing media. An inhibitor, however, prevented secretion of the depolymerase in succinate culture (Mukai *et al.*, 1992). No such inhibitor was found when P(3HB) served as the sole carbon source. The authors suggested that the function of this inhibitor would be to prevent the action of the depolymerases in the absence of P(3HB) as the depolymerases from *P. lemoignei* are secreted constitutively in culture medium containing any carbon sources (Mukai *et al.*, 1992). In addition to the depolymerase, an intracellular 3HB dimer hydrolase and an extracellular 3HB dehydrogenase were also produced constitutively (Delafield *et al.*, 1965a). On media with 3HB as the sole carbon source, enzyme was secreted near the end of the logarithmic phase of growth, during the final 20% increase in the turbidity of cultures before the maximal stationary phase was established as a result of the depletion of the carbon source (Delafield *et al.*, 1965a).

Five different depolymerases were isolated and purified from *P. lemoignei*. These include isozymes A_1 and B_1 (Lusty and Doudoroff, 1966) and their subfractions, A_2 and B_2 (Nakayama *et al.*, 1985); depolymerases C and D (Briese *et al.*, 1994); and P(3HV) depolymerase (Müller and Jendrossek, 1993). Depolymerases A_1 and B_1 exist in single polypeptide chain forms. A serine residue was implicated to be in the active site and disulfide bonds were detected to be required for enzyme activity. The relationship between A_1 and A_2 and that between B_1 and B_2 is not clear, although the two fractions and sub fractions showed many similarities in their physical properties with regards to substrate specificity and products of P(3HB) digestion. Both A_1 and B_1 fractions were highly specific for P(3HB) and hydrolyzed purified granules at approximately the same rate. The trimeric ester of D-3HB also served as substrate for both A_1 and B_1 but the dimer did not. The depolymerases cleaved P(3HB) between the second and third 3HB residues from the free hydroxy terminus, forming dimer and monomer (Nakayama *et al.*, 1985). It was also found that the depolymerases did not hydrolyze P(3HB) to high molecular weight species of oligomers such as tetramer to octamer even at the early stages of the reaction. Instead, the depolymerases constantly produced relatively low molecular weight oligomers (Nakayama *et al.*, 1985). Depolymerase A_1 and B_1 differed with respect to the pathway of P(3HB) digestion and the quantitative yields of end products. The A_1 depolymerase produced very small amounts of trimer. In contrast, the trimer accumulated as the principal product in the B_1 depolymerase until the polymer had virtually disappeared (Nakayama *et al.*, 1985).

P(3HB) depolymerase C and D were found to be related to P(3HB) depolymerase B based on comparisons of the gene products of cloned depolymerase synthesis genes, and may represent isoenzymes of B_1 (Briese *et al.*, 1994) similar to those described by Nakayama *et al.* (1985). The P(3HV) depolymerase had a high specificity for P(3HB), P(3HV) and P(3HB-co-3HV) and hydrolyzed them at comparable rates thus showing the broad substrate specificity of the enzyme (Müller and Jendrossek, 1993). The P(3HV) depolymerase closely resembled A_1 and B_1 depolymerases in physical characteristics.

Synthesis and secretion of the depolymerases from *P. lemoignei* differed according to the carbon substrate present. Synthesis of active P(3HV) depolymerase was dependent on the presence of substrates with odd numbered carbon skeletons. P(3HV) depolymerase activity was absent or very low during growth on P(3HB), 3HB, succinate or acetate. P(3HB) depolymerase A_1 was found to be dependent on catabolism of substrates with even numbered carbon skeletons. The enzyme was absent in P(3HV)- or valerate-grown cultures (Müller and Jendrossek, 1993). P(3HB) depolymerase B_1 appeared not to be regulated by the nature of the substrate as with the other two depolymerases. This enzyme appeared in culture medium at the end of the growth phase on P(3HB), P(3HV), succinate and valerate.

1.3.2.2 *Alcaligenes faecalis*

Tanio *et al.* (1982) reported the isolation of a P(3HB)-depolymerizing bacterium, *Alcaligenes faecalis* T1, from activated sludge and described the subsequent purification and properties of the extracellular P(3HB) depolymerase from the organism. The native enzyme exists in a monomer form (Tanio *et al.*, 1982). Kinetic studies showed the enzyme to have greater affinity for polymers of 3HB higher than trimers. The depolymerase cleaved only the second ester linkage from the hydroxy terminus of the trimer and tetramer (Shirakura *et al.*, 1986). The enzyme also acted as an endo-type hydrolase toward pentamer and higher oligomers of 3HB. Dimers were the main hydrolytic products of P(3HB) depolymerization. Treatment of the P(3HB) depolymerase with trypsin revealed that the enzyme contained a hydrophobic site in addition to the catalytic site (Fukui *et al.*, 1988). The trypsin-treated enzyme did not hydrolyze slightly water-soluble and water-soluble oligomers or P(3HB). In contrast, the intact enzyme showed higher affinity for P(3HB) than for oligomers of 3HB. It was postulated that the higher affinity of the enzyme for P(3HB) was due to the hydrophobic site. The initial interaction of P(3HB) with the hydrophobic site of the depolymerase may cause a conformational change to the enzyme structure. This would, as a consequence, allow a certain portion of the P(3HB) (most likely the hydroxy terminus portion) to come near the catalytic site of the enzyme.

Saito *et. al* (1989) cloned the *A. faecalis* T1 gene for P(3HB) depolymerase into *E. coli* and studied the expression and sequence of the gene. The cloned P(3HB) depolymerase was expressed in *E. coli* as a fully active protein and showed the same characteristics as the native enzyme from *A. faecalis* T1. The amino acid sequence of the depolymerase was deduced from the nucleotide sequence and indicated the presence of a single serine in the peptide fragmented by trypsin treatment. Since this serine was thought to be important in the active site it was believed the depolymerase was similar to serine esterases. Yet the amino acid sequence around the active serine of the depolymerase did not fit any sequence reported for serine esterases.

However, a 91 amino acid segment was found to be slightly homologous to so-called type III homology units of fibronectin (Saito *et al.*, 1993). Fibronectin is a multifunctional extracellular matrix of plasma protein of higher eukaryotes. Similar amino acid sequences have been reported in some types of bacterial chitinases and cellulases, and the P(3HB) depolymerase seems to have an overall similarity to these bacterial extracellular hydrolases.

The growth rate and secretion of P(3HB) depolymerase by *A. faecalis* T1 was found to be dependent on the carbon source in the growth medium (Zhang *et al.*, 1992). When *A. faecalis* T1 was grown in medium containing P(3HB) as the sole carbon source, the activity of the depolymerase was maximal at the end of exponential growth (30 h)

(Tanio *et al.*, 1982). Although Tanio *et al.* (1982) detected no enzyme secretion when 3HB or its dimeric ester served as the carbon source, studies by Shirakura *et al.* (1983) and Zhang *et al.* (1992) detected enzyme secretion. When glucose served as the carbon source, the doubling time was 10 h after a 60-h lag period and as *A. faecalis* T1 grew exponentially, P(3HB) depolymerase was secreted (Zhang *et al.*, 1992). In contrast, when succinate served as the carbon source, the culture grew exponentially with a doubling time of 1-h after a 2-h lag period, yet no P(3HB) depolymerase was secreted. Further analysis revealed that the succinate-grown cells did produce a depolymerase in the cytoplasm but lacked the ability to translocate it across the inner and/or outer membrane (Zhang *et al.*, 1992). The P(3HB) depolymerase activity was localized to the membrane fractions of succinate- and glucose-grown cells, and Zhang *et al.* (1992) proposed the enzyme was attached to the membrane through a hydrophobic interaction.

In basal salt medium supplemented with P(3HB) as sole carbon source, a trace amount of P(3HB) depolymerase may leak into the medium or the enzyme localized on the surface of the cells may hydrolyze P(3HB) thus liberating 3HB. 3HB can act as a signal for P(3HB) depolymerase secretion. *A. faecalis* T1 also possesses an extracellular oligomer hydrolase (Shirakura *et al.*, 1983) that has a high affinity for water-soluble oligomeric esters of P(3HB). The general degradation pathway of P(3HB) by *A. faecalis* T1, therefore, is the hydrolysis of the polymer by the P(3HB) depolymerase to water-soluble 3HB dimers or higher water-soluble oligomers which are then hydrolyzed to monomeric forms by the oligomeric hydrolases. These monomers can then be taken up and utilized by the bacterium as a carbon source.

1.3.2.3 *Comamonas* spp.

A novel type of P(3HB) depolymerase was purified from a *Comamonas* sp. and characterized by Jendrossek *et al.* (1993). The organism grew on organic acids such as succinate, pyruvate, lactate, acetate, 3HB or P(3HB) and on complex media. The organism did not grow on sugars or related compounds.

The synthesis of active P(3HB) depolymerase was repressed in the presence of most substrates. The depolymerase was secreted, however, in medium containing 0.1% (w/v) P(3HB) and the maximal specific activity occurred during mid-exponential growth phase. Depolymerase activity was present only in the supernatant of the culture broth, no activity was detected in the cytoplasm or membrane fraction of cell extracts.

The native P(3HB) depolymerase consisted of one single polypeptide chain with essential disulfide bonds as indicated by inhibition studies (Jendrossek *et al.*, 1993). The enzyme was found to have no dependence on heavy metal ions, reduced sulfur groups or active serine residues for activity.

The depolymerase was highly specific for the hydrolysis of PHA. Highest activity was with P(3HB); activity with P(3HV) was 1.5% as compared with P(3HB) as substrate. Activities with P(3HB-co-3HV) were in between those of P(3HB) and P(3HV). Poly(3-hydroxyoctanoate) P(3HO) was not significantly hydrolyzed. The main hydrolysis products of the polymers were monomer units. The depolymerase did not hydrolyze DNA, albumin, polylactids or substrates for lipases.

A P(3HB) depolymerase was also purified from *Comamonas testosteroni* which had been isolated from sea water (Mukai *et al.*, 1993b). As with the other depolymerases discussed, the activity of the enzyme was maximal at the end of the logarithmic growth phase. The pH range for optimal enzyme activity was between 9.5 to 10.0 in glycine-NaOH buffer (Mukai *et al.*, 1993b). Similarly, the pH for optimum enzyme activity for *Comamonas* sp. was 9.4 in 100 mM Tris-HCl buffer (Jendrossek *et al.*, 1993). The depolymerase from *C. testosteroni* also had a broad specificity for PHAs with various compositions.

1.3.2.4 *Pseudomonas pickettii*

Pseudomonas pickettii was isolated as a P(3HB)-degrading organism from air (Yamada *et al.*, 1993). The secretion of the *P. pickettii* depolymerase was dependent on the carbon source added, and of the substrates tested, only P(3HB) and 3HB were able to induce enzyme secretion. The best substrate for growth was succinate, but no depolymerase activity was detected in culture supernatant. The depolymerase activity was maximal at the end of exponential growth phase. The optimum pH range and temperature for maximal activity was 5.0 to 6.0 and 40°C respectively. Inhibition studies revealed a serine residue in the active site and a requirement for disulfide bonds for activity.

1.3.2.5 *Pseudomonas fluorescens* GK13

Schirmir *et al.*(1993) described the isolation and characterization of a P(3HO)-degrading bacterium and the purification and properties of a novel PHA depolymerase. An extensive screening process yielded 26 P(3HO)-degrading bacteria isolated independently from various soils, lake water and activated sludge. The most efficient P(3HO)-degrading bacteria belonged to the fluorescent pseudomonads, and one of these, chosen for further study, was identified as *Pseudomonas fluorescens* GK13.

This organism grew on P(3HO), poly(3-hydroxydecanoate-co-3-hydroxyoctanoate) P(3HD-co-3HO), ethanol, complex media, sugars and most organic acids with a doubling time on P(3HO) of 2 h and on P(3HD-co-3HO) of 24 h. The secretion of active P(3HO) depolymerase was found to be substrate dependent. Little or no activity was detected when *P. fluorescens* GK13 grew on glucose, alanine, acetate, succinate or acetate plus P(3HO). Low activities were detected when medium chain length fatty acids such as decanoate served as carbon source. The highest depolymerase activities were found at the end of the exponential growth phase on lactate, pyruvate, 3-hydroxy acids (3HA) such as 3HB, 3HO and 3HD and the PHAs, P(3HO) and P(3HD-co-3HO). The high expression of P(3HO) depolymerase depended, therefore, on the presence of 3HA or similar compounds to serve as inducers as well as the starvation of the cells for carbon. The presence of non-inducing substrates such as acetate along with P(3HO) repressed the synthesis of active P(3HO) depolymerase. It is not known why pyruvate and lactate would induce P(3HO) depolymerase synthesis whereas alanine or glucose which are metabolized via pyruvate, would not induce the synthesis of the enzyme.

The native enzyme consisted of one single polypeptide chain. The optimum pH range for activity was 6.5 to 9.3 and the optimum temperature range was 30 to 32°C. The enzyme was specific for the hydrolysis of P(3HO) and P(3HD-co-3HO). The main product of P(3HO) hydrolysis was determined to be dimers. The P(3HO) depolymerase did not significantly hydrolyze P(3HB), P(3HV) or the copolymer of these two. No hydrolytic activity was detected with DNA, casein or substrates for lipases such as Tween 80 or triolein.

No dependence on metal ions, reduced thiol groups, active serine residue or essential disulfide bonds for its activity was found. In summary, the depolymerase from *P. fluorescens* GK13 has a different protein structure and reaction mechanism from the depolymerase of *A. faecalis* T1, *Comamonas* spp. and *P. lemoignei*.

1.3.2.6 *Ilyobacter delafieldii*

Ilyobacter delafieldii represents the first isolated anaerobic bacterium that can degrade exogenous P(3HB) (Janssen and Harfoot, 1990). This bacterium grew on crotonate, 3HB, P(3HB), pyruvate and lactate. Sulfate, sulfur, thiosulfate and nitrate were not used as terminal electron acceptors when crotonate served as the carbon source.

An extracellular P(3HB) depolymerase was secreted by *I. delafieldii* as indicated by clear zones around colonies in P(3HB) agar shakes. Agar shakes were prepared by adding 0.75 g purified agar per liter of medium. P(3HB) was added at 0.05 g per 10 mL agar shake. Most of the depolymerase activity was found to be associated with P(3HB) granules in liquid cultures (Janssen and Schink, 1993). No depolymerase activity was detected when the other growth-supporting carbon sources, crotonate, 3HB, lactate or pyruvate, were tested.

When P(3HB) served as the carbon source, acetate and butyrate were detectable as the fermentation products. When acetate and butyrate production and cell growth slowed and halted, 3HB began to accumulate. It was assumed 3HB was formed as an intermediate of P(3HB) degradation, but did not become detectable until growth on P(3HB) had ceased. Production of 3HB was, therefore, not directly linked to cell activity (Janssen and Harfoot, 1990).

The limited metabolic capacity of *I. delafieldii* and its ability to grow on P(3HB) suggested that this organism may be specialized to degrade this polymer upon death and lysis of other bacterial cells. The fact that this organism was isolated from anaerobic mud where detritus collects lends support to this hypothesis.

1.3.2.7 Fungi

The production and secretion of PHA depolymerases are not restricted to prokaryotes. There have been reports that eukaryotes, namely fungi, are able to biodegrade exogenous PHA.

One study isolated two fungi, *Penicillium simplicissimum* and *Eupenicillium* sp., from various soils (McLellan and Halling, 1988). These organisms were found to be able to secrete P(3HB) depolymerases that were active at very acidic pH values such as 3. The main product of P(3HB) hydrolysis was found to be the 3HB monomer. As with the bacterial depolymerases, the hydrolysis of P(3HB) by the fungal depolymerases did not yield any P(3HB) oligomers. Thus the depolymerase may act in an exo-fashion, releasing monomer units from the ends of polymer chains, or other unidentified enzymes may rapidly degrade any oligomers produced.

The P(3HB) depolymerase isolated from the fungus *Penicillium funiculosum* was found to have many properties similar to, but also different from, prokaryotic P(3HB) depolymerases (Brucato and Wong, 1991). The *P. funiculosum* depolymerase had maximal production and activity at the late growth stage. The enzyme was found to exist as a single polypeptide chain as do bacterial depolymerases. One free sulfhydryl and four disulfide bonds were found in the enzyme. Inhibition studies indicated the presence of an essential serine and a carboxylic acid in the active site. The depolymerase may be a serine esterase similar to the depolymerase from *P. lemoignei*. Unlike *A. faecalis*, the hydrophobic site on the *P. funiculosum* depolymerase may be located near or at the active site. The major difference between the *P. funiculosum* depolymerase and the depolymerases from prokaryotes is that the *P. funiculosum* depolymerase is glycosylated.

1.3.3 Degradation of Films by Depolymerases

The enzymatic degradation of PHAs in the form of films is highly dependent on a variety of factors such as composition and degree of crystallinity of the PHA. Several studies, discussed in the following sections, have addressed these factors and deduced the mechanism of PHA film degradation by bacterial depolymerases.

1.3.3.1 Effect of PHA Structure and Composition on Biodegradation Rates

The effect of composition of PHA samples on hydrolytic and enzymatic degradation was examined by Doi *et al.* (1990b). Seven different PHA film samples of various molecular weights and compositions (pure P(3HB) and P(3HB-co-3HV)) were prepared.

During non-enzymatic hydrolytic degradation, carried out at 55°C in 0.01 M phosphate buffer, the weights of all films remained unchanged. However, the molecular weights decreased with time. There was an induction period when no change in molecular weight occurred and the film thickness of the samples increased. This suggested that water had permeated the polymer matrix and the induction period was necessary to allow full permeation of water (Doi, 1990). A similar hydrolytic degradation study by Holland *et al.* (1987) also suggested that a gradual diffusion of water into the bulk of film accompanied by progressive chain scission within the polymer matrix occurred. The rate of molecular weight decrease was affected by the composition.

In contrast, during enzymatic degradation with the purified P(3HB) depolymerase from *A. faecalis* T1 in a 0.1 M phosphate buffer at 37°C, the molecular weights remained unchanged but the weight of the films decreased with time. The rate of film erosion strongly depended on the composition of the polymer with copolymers of P(3HB-co-4HB) degrading faster than P(3HB-co-3HV) films. The rapid erosion of P(3HB-co-4HB) films by the depolymerase may be due to the fact that the 4HB units are less sterically bulky than 3HB and 3HV units, thus allowing easy access of the enzyme towards the ester groups of the polymer chains. The *A. faecalis* depolymerase, therefore, hydrolyzes only the polymer chains in the surface layers of the films and the degradation proceeds via the surface and not by internal random chain scission as in simple hydrolysis.

Although Doi *et al.* (1990) did not find a correlation between rates of enzymatic or hydrolytic degradation and the crystallinities of the films, subsequent studies by other researchers did. Kumagai *et al.* (1992) found that the rate of P(3HB) biodegradation by the *A. faecalis* depolymerase decreased with an increase in crystallinity. The molecular weight of the films remained unchanged and so, in accordance with findings by Doi *et al.* (1990), the authors suggested that polymer erosion proceeded via surface dissolution.

A study by Nishida and Tokiwa (1993) also showed that crystalline regions in PHA films retarded microbial degradation and this suggested that amorphous regions were degraded selectively or preferentially. SEM observations of films degraded by soil isolates revealed that degradation took place at the surface layer and the inside portions of the samples were apparently unchanged. As well, spherical holes on surfaces were observed and changes in crystal structure of P(3HB) significantly influenced the size, number and even the formation of these holes.

Microbial degradation of P(3HB) is thought to proceed in at least two manners. One is the preferential degradation of amorphous regions at the surface layer leaving crystalline lamellae in relief. This is assumed to be due to homogeneous enzymatic action over the entire surface of the film by diffused extracellular depolymerases. The other manner is by nonpreferential degradation when there is a localized intense enzymatic action with colonization of the degrading bacterium. This results in the formation of spherical holes on the film surface. Nishida and Tokiwa (1993) put the hypothesis forward that the change of crystal structure of P(3HB) affects the colonization and physiological action of the degrading bacterium on the P(3HB) surface. This can be explained by assuming there may be several points prone to degradation in the crystalline region and that would serve as sources of carbon. If the quantity of the carbon source at a point is sufficient for the formation of a colony, the degrading bacterium may form a colony at this point, and consequently, a spherical hole. But, if the carbon supply is insufficient owing to slow degradation no colonization and no spherical holes will be found.

In order to obtain a better understanding of the relationship between polymer properties and susceptibility toward depolymerase activity, Jesudason *et al.* (1993) looked at the degradation of synthetic P(3HB) fractions of varying crystallinity and isotactic diads. Synthetic P(3HB) fractions, synthesized from racemic ß-butyrolactone using an *in situ* trimethyl aluminum-water catalyst, were prepared and divided into 3 fractions: (1) high crystallinity (67%) and 88% isotactic diads fraction; (2) medium crystallinity and 63% isotactic diads fraction; (3) low crystallinity (9%) and 54% isotactic diads fraction. The biodegradation of these samples were compared to the biodegradation of bacterial P(3HB) by the depolymerase from *A. faecalis* in phosphate buffer at 37°C.

Results showed that the synthetic P(3HB) with the highest crystallinity and percentage isotactic diad content had hardly been degraded after 50 h of incubation when the experiment was stopped. The synthetic P(3HB) sample with medium crystallinity and percentage isotactic diad content lost mass continuously and steadily throughout the experiment. The sample with the lowest crystallinity and percentage isotactic diad content lost mass rapidly at first and then slowly as the experiment progressed. The samples of bacterial P(3HB) showed the most rapid degradation of all the samples.

Since the degree of crystallinity of the bacterial P(3HB) was comparable to the crystallinity of the high percentage isotactic diad synthetic P(3HB), the degree of crystallinity was not a determining factor in the enzymatic degradation of the films in this case. The main difference between bacterial P(3HB) and the synthetic P(3HB) was that the bacterial P(3HB) was comprised only of [R]-3HB stereoblocks, while the synthetic P(3HB) was comprised of both [R] and [S] stereoblocks due to the nonspecificity of the synthesis reaction.

It has been established that bacterial depolymerases have a specificity for [R] linkages in P(3HB) (Shirakura *et al.*, 1986). The *A. faecalis* T1 depolymerase hydrolyzes P(3HB) at the hydroxyl end groups and can hydrolyze oligomers as small as trimers. It therefore seems that a sequence of 3 [R] repeats from the hydroxyl terminus would serve as the template for enzymatic hydrolysis. The enzyme would then proceed to cleave ester linkages bordering accessible [R] stereoblocks along the polymer chains and proceed throughout the sample as more surface area is exposed. The presence of the [S] stereoblocks in the synthetic polymer would prevent the depolymerase from degrading an accessible [R] stereoblock exposed at the film surface.

The synthetic polymer of low crystallinity and percentage isotactic diad showed an abrupt halt in degradation after an early rapid degradation. This was most likely due to a lack of sufficiently long blocks of [R] stereoblocks along the chain. The low proportion of 3 consecutive [R] sequences initially present in a nonisotactic sample would then consist mainly of [S] butyrate ester linkages and would not be susceptible to the depolymerase. The synthetic polymer of medium crystallinity and 63% isotactic diad had the greatest weight loss. This reflected an optimal balance between mobile accessible chains as measured by crystallinity and stereochemistry.

The effect of crystallinity on the surface accessibility for polymer degradation was demonstrated by Jesudason *et al.* (1993) by comparing the enzymatic degradation of freeze-dried P(3HB) granules and non-dried P(3HB) granules that had been isolated by the hypochlorite method. The depolymerase hydrolyzed the non-dried granules faster than the freeze-dried granules. The polymer chains in the non-dried granules remained primarily in a mobile state and this mobility allowed greater accessibility of enzymes to the chains compared to dried granules. The freeze-dried granules crystallize upon drying and so the polymer chains became less mobile and less accessible to the depolymerase.

1.3.3.2 Specificity and Kinetics of Enzymatic PHA Film Degradation

Mukai *et al.* (1993c) compared the substrate specificities of different PHA depolymerases and lipases on the hydrolysis of microbial and synthetic PHA samples. The lipases from the eukaryotic and prokaryotic sources were able to degrade the synthetic PHA samples (which consisted of poly(3-hydroxypropionate), poly(5-hydroxyvalerate) and poly(3-hydroxyhexanoate) but not the bacterial P(3HB) sample. In contrast, the microbial PHA depolymerases were able to hydrolyze the P(3HB) films. Poly(3-hydroxypropionate) was also able to serve as a substrate, however poly(5-hydroxyvalerate) and poly(3-hydroxyhexanoate) were not degraded.

It has been well established that enzymatic degradation of polymers proceeds as surface erosion by depolymerases, however little is known about the kinetics of this reaction. A study was carried out, therefore, to determine the kinetics and mechanism of enzymatic degradation on P(3HB) film surfaces by using 3 PHA depolymerases from *A. faecalis*, *P. pickettii* and *C. testosteroni* (Mukai *et al.*, 1993a).

Because the *A. faecalis* depolymerase has both a catalytic site and a hydrophobic domain as the binding site, a two-step reaction mechanism was deduced for the degradation of a film surface by the depolymerase. Firstly, the enzyme adsorbs onto the surface via the binding site and secondly the catalytic site mediates the hydrolysis of polymer chains into water-soluble products. A kinetic model for such heterogeneous enzymatic reactions was proposed.

The model takes into account the total number of adsorption points per unit surface area of P(3HB) film and the number of enzyme molecules adsorbed per unit area. The model shows that the rate of hydrolysis increases proportionally with enzyme concentration at low concentrations of enzyme. The rate of hydrolysis reaches a maximum at the concentration of enzyme such that the adsorption constant is equal to one. At higher concentrations of enzyme, the rate is inversely proportional to enzyme concentration and becomes almost zero. The reason for this observation is that at high concentrations of enzyme the majority of the enzyme molecules are adsorbed onto the polymer surface by the hydrophobic domain and block the catalytic site from coming into contact with the polymer chains for hydrolysis. Indeed, the rate of enzymatic degradation was observed to increase to a maximum value with an increase in concentration of PHA depolymerase. This was followed by a gradual decrease in rate of degradation as concentration of depolymerase continued to increase. The depolymerase from *C. testosteroni* was most sensitive to enzyme concentration with regards to rate of degradation.

The rate constants for P(3HB) hydrolysis were almost identical for the three PHA depolymerases tested. This suggested that the properties of the active site were similar among the three depolymerases. From individual studies described in section 1.3.2, the active sites of the various depolymerases are indeed alike in the fact that there is usually a serine residue.

The adsorption equilibrium constant (K) of the enzymes, on the other hand, differed depending on the organism with the K values decreasing in the following order: *C. testosteroni* > *A. faecalis* > *P. pickettii*. The order of size of K was in agreement with the determined hydrophobicities of the enzymes suggesting that the properties of the binding domains of the enzymes were strongly dependent on the type of PHA depolymerase. *C. testosteroni*, which had the highest K value, was isolated from sea water, *A. faecalis* was isolated from activated sludge and *P. pickettii*, which had the lowest K value, was isolated from laboratory atmosphere. It may be possible, therefore, that the PHA depolymerase of bacteria in aqueous environments possess binding domains with a high hydrophobicity to enable adherence to the surface of P(3HB) films.

1.3.4 *In Vivo* and *In Situ* Tests on PHA Biodegradation

Only recently have studies been reported on the biodegradation of PHAs by microbial consortia collected from the environment and brought to the laboratory or by direct introduction of the PHAs into the specific environment being tested. The majority of the samples have been from aerobic environments and no specific attempts were made to subject the samples to anaerobic conditions.

1.3.4.1 Soils

The biodegradation of P(3HB) and P(3HB-co-10%3HV) films in five different soils under laboratory conditions was analyzed by Mergaert *et al.* (1993). They used injection molded, dog bone shaped tensile polymer test pieces, 83 mm long, 2 mm thick and weighing approximately 1.75 g. The test pieces were buried in 1 kg of the following soils: sandy (pH 6.5), clay (pH 7.1), loamy (pH 6.3), hardwood forest (pH 3.9) and pinewood forest (pH 3.5) soil. During incubation of up to 200 days at a constant temperature of 15, 28 or 40°C, test pieces were removed and molecular weights of the films and mechanical properties were determined. A sterile control was run in parallel to the soil experiments in order to measure non-enzymatic hydrolysis of the films.

There were no weight losses of PHA films by non-enzymatic hydrolysis. The molecular weight did not change when hydrolysis was carried out at low temperatures, however, there was a significant reduction in molecular weight at higher temperatures (40-50°C). These results are in accordance with Doi *et al.* (1990b) in that during hydrolysis the weight remains the same but the molecular weight decreases due to random chain scission.

The decrease in molecular weight at 55°C affected the tensile strength of the film but not the stress at break and Young's modulus.

All the test pieces incubated in soil lost weight, but the rate at which weight loss occurred varied widely (from 0.03 to 0.64% weight loss/day) depending on incubation temperature, soil type and composition of the polymer. Higher temperatures of 40°C enhanced biodegradation of the test samples to different extents and this was highly dependent on soil type. In acidic soils (hardwood and pinewood forest soils), the P(3HB-co-3HV) films degraded much faster than the P(3HB) films. In neutral soils (sandy, clay and loamy soils), however, the degradation rates of P(3HB) and P(3HB-co-3HV) were similar. P(3HB-co-3HV) films may have been degraded faster since they contain a larger proportion of amorphous regions than do P(3HB) films. The effect of pH is not known.

Unlike the weight loss, molecular weight of the films decreased in a similar way in all of the soils, and was affected by incubation temperature such that at temperatures of 40°C or above there was a significant decrease in molecular weight. This decrease occurred to the same extent in sterile buffer when incubated at 40°C suggesting that in the soil experiments, hydrolysis of the polymer probably occurred independently of any biodegradation. This did not seem to be a surface phenomenon since no relevant differences in molecular weights between samples taken from centers and from surfaces of the test pieces were observed. It was concluded that biodegradation of PHA films in soil seems to occur on at least two levels: 1) throughout the polymer sample by simple hydrolysis affecting the molecular weight but not the weight of the polymer, and 2) at the surface by depolymerases which degrade the polymer to low-molecular weight fragments that are then used by microorganisms resulting in a decrease in polymer weight.

The elongation to break values of PHA tensile test pieces decreased approximately 2- to 3-fold after 150 days for all incubation periods and in all soils. These values were much more affected in soil than sterile buffer. Therefore, in addition to the decrease in molecular weight, surface roughness as a result of degradation may also have contributed to loss of strength. Film surfaces were observed by SEM to change from matte to a progressively rougher texture with biodegradation occurring on all the surfaces.

The dominant PHA-degrading microorganisms found in the soil were isolated and identified. The spectrum of microorganisms included Gram negative and positive bacteria, actinomycetes and molds. Acidic soils contained relatively few P(3HB)-degrading Gram positive bacteria. A wide range of different polymer-degrading microorganisms were isolated from soils at mesophilic temperatures, but only a few species were found at higher temperatures (40°C and higher) that could degrade PHA. It was not clear whether the differences in polymer degradation rates in the different soils were attributable to the differences in composition of the dominant degrading microorganisms.

P(3HB) and P(3HB-co-3HV) films buried in highly calcareous forest soils become colonized mainly by fungi and actinomycetes (Lopez-Llorca *et al.*, 1993). SEM pictures revealed the formation of fungal hyphae after 15 days of incubation. After 45 to 90 days in the soil, the films showed clear signs of degradation. Deep tracks associated with the fungal hyphae were observed on the film surface. Actinomycete filaments were seen later during the incubation period. Thus, many microorganisms in soils can degrade P(3HB).

1.3.4.2 Activated Sludge

The effects of inoculum size, pH and agitation of activated sludge cultures on the biodegradation of P(3HB) films were investigated (Briese *et al.*, 1994). Inoculum size was found to have no influence on the degradation rate as measured by loss of film weight. The pH, on the other hand, exerted a significant influence on the degradation rate. At an optimum pH of 7.5 to 8.0, films were degraded with 100% weight loss within 12 weeks. This pH range is usually the pH optima of P(3HB) depolymerases. At pH values below 6 and above 9 less than 10% of the polymer was degraded after 12 weeks.

The degradation rates in constantly shaken cultures were significantly higher than those of non-agitated and daily shaken flasks. The increased oxygen dissolution due to the constant agitation would result in higher metabolic rates and consequently higher rates of polymer degradation.

In situ tests looking at the biodegradation of PHAs versus several blends of degradable polymers and polyolefins in activated sludge were performed by Gilmore *et al.* (1993). The polyolefin polymer samples were blends of polypropylene (PP) and 6% corn starch, linear low density polyethylene (LLDPE) and 12% cornstarch, and LLDPE and 30% polycaprolactone (PCL). The solution cast film PHA samples included P(3HB-co-26.5%3HV) and P(3HO-co-12%3HH). All of the polymers were cut into dog bone shaped films and either attached to sticks and submerged in the activated sludge or encased within mesh bags and wire baskets and submerged so that all sides of films were exposed.

The samples were suspended in the aeration area of the activated sludge tanks where substrate concentration and, therefore, microbial activity were expected to be highest. The films were then periodically sampled, cleaned and tensile properties and molecular weights determined.

Of all the samples, only P(3HB-co-26.5%3HV) lost significant amounts of weight (over one-half of its initial mass after 138 days of incubation). As well, the tensile strength showed a large decrease only with the P(3HB-co-26.5%3HV) samples. No significant changes in either percentage elongation to break or tensile strength occurred with the starch/polyolefin blends. The sterile P(3HB-co-26.5%3HV) control had no weight loss nor changes in mechanical properties so the reduction in weight and strength in the P(3HB-co-26.5%3HV) samples was attributed to biodegradation.

There were no significant changes in molecular weight for P(3HB-co-26.5%3HV) samples in either native or sterilized activated sludge, therefore no chemical hydrolysis occurred throughout the bulk of the samples. The thickness of the P(3HB-co-26.5%3HV) samples decreased significantly with time of incubation as a result of surface erosion. This would be expected if the primary mechanism of degradation was through enzymatic attack known to be a surface phenomenon.

The results clearly showed that P(3HB-co-26.5%3HV) was degraded biologically and not chemically. Higher temperatures increased the rate of degradation by stimulating microbial metabolism and enzymatic activity. P(3HO-co-12%3HH) was less biodegradable than P(3HB-co-26.5%3HV). This was probably due to its high hydrophobicity resulting from the longer alkyl side chains of its monomer constituents. Microorganisms have difficulties colonizing the surface of this hydrophobic material in an aqueous environment.

The reason why the polyolefin blend films did not show any weight loss may be due to the inaccessibility of the starch or polycaprolactone encased in the polyolefin matrix. The use of degradation enhancing additives such as photosensitizing agents or prooxidants would not likely have any effect on the rate of biodegradation. The sludge would block UV radiation from the sun and the heat dissipation by waste water would have limited prooxidant effectiveness.

1.3.4.3 Compost

The same polymers that were used in the activated sludge experiment (Gilmore *et al.*, 1993) were also exposed to a municipal leaf composter and the rates of biodegradation measured (Gilmore *et al.*, 1992). The dog boned shaped films were attached in duplicate onto sticks inserted approximately 0.6 m into compost windrows. Once each month for 6 months, a sample stick was sacrificed and the polymer samples analyzed for tensile properties, thickness, molecular weight, and bacterial colonization and morphology by SEM.

After 2 months of exposure, SEM pictures of P(3HB-co-26.5%3HV) films showed the surface had changed from being flat and featureless to being extensively pitted and grooved. The probable cause of these pits and grooves was due to depolymerase activity from colonies of fungi and bacteria observed on the films by SEM. Despite the fact that both bacteria and fungi associated with the polymer, screening tests showed that only the fungi had PHA depolymerase activity. In similar studies, Matavulj and Molitoris (1992) found a high number of fungi associated with Biopol samples in composts while Mergaert *et al.* (1992) found few fungi but mostly bacteria and streptomycetes degrading PHAs in a compost.

Gilmore *et al.* (1992) reports a significant correlation between loss of weight of P(3HB-co-26.5%3HV) samples in compost to time of exposure. Since no physical abrasion mechanism occurred during exposure of the films to the compost (the films were intact and coated with a biofilm) and the sterile control samples showed no weight loss, it was concluded that biological activity was required for the weight loss to occur.

The extensibility was decreased by nearly one-half after 1 month of exposure and this dropped along with strength to zero after 4 months. However, the sterile control samples became very brittle with no measurable extensibility or strength by the end of the 6-month incubation period. The molecular weights of the samples decreased steadily during the first 4 months with no significant changes in polydispersity. This decrease in molecular weight was correlated to loss of tensile strength. The compost temperature ranged from 42 to 52°C during the test period. Similar decreases in molecular weight were observed in P(3HB-co-26.5%3HV) samples incubated for 6 months at 55°C in a sterile control environment, thus implicating abiotic hydrolysis as the cause of this decrease.

P(3HB-co-26.5%3HV) was, therefore, degraded by a combination of biological and chemical mechanisms. Hydrolytic degradation reduced the molecular weight of the polymer increasing the number of chains available for enzymatic attack by microorganisms.

No significant degradation occurred with the starch/LLDPE blends. Microbial colonization was not significant as compared to PHB/V samples. Some starch granules in the blends were observed to be partially degraded but in the blends containing less than 40% starch, the starch was likely encased in the polyolefin (LLDPE) and inaccessible to microbial enzymes. This was also the case with the PCL/LLDPE blends; the biodegradable PCL when blended with non degradable LLPDE became inaccessible to microbial enzymes. Not more than 13% of the total PCL was determined to be removed from the blends.

1.3.4.4 Aquatic - Marine

PHAs were found to be biodegradable in seawater (Doi *et al.*, 1992, Mergaert *et al.*, 1992; Lopez-Llorca *et al.*, 1994). Surface erosion (loss of weight) occurred in P(3HB), P(3HB-co-3HV) and P(3HB-co-4HB) samples submerged in the water and was dependent on the temperature, with increased biodegradation rates at higher temperatures (Doi *et al.*, 1992). The temperature of the water ranged from 13°C to 26°C during this 1-year study. The molecular weight of the samples decreased slightly as the surface erosion proceeded and the samples became brittle, but the polydispersities remained the same. Simple hydrolysis was ruled out as a mechanism of polymer degradation in the marine environment since a killed control showed no weight loss with time (Doi *et al.*, 1992). Doi *et al.* (1992) were able to isolate a streptomycetes strain from seawater able to degrade the polymers.

1.3.4.5 Aquatic - Freshwater

A study was conducted to determine quantitatively the life span of Biopol bottles in a natural aquatic ecosystem (Brandl and Püchner, 1990). The bottles were positioned exactly at the sediment water interface and at different water depths in a Swiss lake and the biodegradation of the bottles monitored.

After 250 days incubation, the dry weight of the bottles had decreased approximately 7% from the original weight. Assuming a linear time course, the life spans of the bottles in the lake were calculated to be approximately 10 years. The life spans increased with increasing water depth as temperature, oxygen content and hydrostatic pressure were all connected to depth of water and affected rates of biodegradation. The calculated life spans were maximal values, as degradation rate was a function of surface area available for microbial attack and the surface area of the bottles increased with time due to the formation of cavities and pores.

Strips of Biopol submerged in the Danube river lost 29% of the initial weight after 1 year (Matavulj *et al.*, 1993), whereas PHB and its copolymers degraded very slowly in a freshwater pond (Mergaert *et al.*, 1992). Lopez-Llorca *et al.* (1994) also observed faster rates of PHA biodegradation (as measured by weight loss) in organically polluted waters than in cleaner waters. The relative rates of PHA degradation were, therefore, highly dependent on the environment used since the composition of the population of degrading microorganisms and their enzymes varied.

1.4 An Introduction to Anaerobic Environments

Anoxic or anaerobic conditions develop when all of the oxygen has been utilized and oxygen is no longer available in free form. Microorganisms under these conditions must use alternate electron acceptors for carrying out redox metabolic reactions. Fermentation also occurs with organic compounds serving as terminal electron donors. The most common terminal electron acceptors for anaerobic respiration are nitrate, ferric iron (Fe(III)), sulfate and CO_2.

Anaerobic nitrate reduction can occur in two different manners; by respiratory denitrification and dissimilatory nitrate reduction to ammonium (Tiedje, 1988). The majority of nitrate reduction is carried out by respiratory denitrification and is of concern in this study. During anaerobic respiration, certain bacteria reduce nitrogenous oxides (principally nitrate and nitrite) to dinitrogen gases, N_2O and N_2 (Tiedje, 1988). This process is coupled to electron transport phosphorylation. Microorganisms capable of dissimilatory nitrate reduction are widely distributed in nature including soils, fresh water, marine waters, sediments, waste treatment systems and animal gastrointestinal tracts.

In a recent review, Lovley (1991) stated the reduction of Fe(III) to be the most important chemical change that takes place in the development of anaerobic soils and sediments and greatly influences the biogeochemical cycles of carbon and many metals. In addition, Fe(III) may be the most abundant potential electron acceptor for organic matter oxidation in many soils, aquatic sediments and aquifers (Lovley, 1991). In dissimilatory Fe(III) reduction, bacteria use Fe(III) as an external electron acceptor during metabolism reducing Fe(III) to ferrous iron (Fe(II)).

Sulfate reduction is carried out by a group of prokaryotes termed the sulfate-reducing bacteria or SRB. SRB use sulfate ions as oxidizing agents for the dissimilation of organic matter (Singleton, 1993). Sulfide ions are released into the environment as end products. SRB are obligate anaerobes and are very widely distributed including such diverse environments as soils; fresh, marine and brackish waters; hot springs; oil and natural gas wells; esturine muds; sewage and corroding iron (Postgate, 1979).

Methanogens reduce CO_2 to methane using H_2 produced by other bacteria and can also cleave acetate to methane and CO_2 (Mackie *et al.*, 1991). The limited substrate utilization indicates that methanogens require synergistic associations with fermentative bacteria who degrade biopolymers and organic matter. Methanogens account for about 10% of the total microflora of the strictly anaerobic bacteria that make up the dominant population in sewage digestors (Garcia, 1990).

The relative energetics of different electron acceptor reactions are given by the following thermodynamic equations in which H_2 is chosen as the common electron donor (Ferry, 1992 and Thauer *et al.*, 1977).

$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$	$\Delta G^{\circ\prime} = -1121$ kJ/reaction	Eqn. 1.1
$2Fe^{3+} + H_2 \rightarrow 2Fe^{2+} + 2H^+$	$\Delta G^{\circ\prime} = -228.3$ kJ/reaction	Eqn. 1.2
$SO_4^{2-} + 2H^+ + 3H_2 \rightarrow H_2S + 3H_2O$	$\Delta G^{\circ\prime} = -151.9$ kJ/reaction	Eqn. 1.3
$HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O$	$\Delta G^{\circ\prime} = -135.6$ kJ/reaction	Eqn. 1.4
$CH_3COOH \rightarrow CH_4 + CO_2$	$\Delta G^{\circ\prime} = -31.0$ kJ/reaction	Eqn. 1.5

The large negative free energy of reaction for nitrate reduction (Eqn. 1.1) indicates the reaction is more favorable than ferric iron, sulfate, carbonate or acetate reduction (Eqns. 1.2 - 1.5 respectively). The energy yield from the reduction of nitrate coupled to an ATP-generating system would be greater than from the other systems.

Under steady-state conditions, the different anaerobic terminal electron-accepting processes in anaerobic sediment segregate into distinct zones in which one H_2-consuming reaction predominates (Lovley and Goodwin, 1988). H_2 is known to be an important intermediate in microbial oxidation of organic matter coupled to reduction of terminal electron acceptors (Lovley *et al.*, 1994). SRB can metabolize H_2 at lower concentrations than methanogens and when the concentration of sulfate as a terminal electron acceptor is not limiting, SRB lower the H_2 concentration so that methane production from H_2 is thermodynamically unfavorable (Lovley and Goodwin, 1988). Likewise, Fe(III)-reducing bacteria can metabolize H_2 at much lower concentrations than SRB and methanogens and when Fe(III) is abundant, Fe(III)-reducing bacteria produce thermodynamically unfavorable conditions for sulfate-reduction and methanogenesis even when active populations of SRB and methanogens are present. Nitrate-reducing bacteria can metabolize H_2 at even lower concentrations than Fe(III)-reducing bacteria, SRB and methanogens (Lovley *et al.*, 1994).

1.5 Objectives

The literature review showed that the aerobic fate of PHAs has been the focus of the majority of the research done on the biodegradation of these polymers. Very little is known, however, on the anaerobic biodegradation of PHAs. The study of the anaerobic biodegradation of PHAs is important since it would establish that the polymers are indeed biodegradable under conditions that exist in most garbage disposal and burial sites, if sufficient moisture is present (Palmisano *et al.*, 1993). With most civic landfills reaching their capacities, the managed biodegradation of waste plastic made from PHAs by anaerobic digestion may be a feasible solution to the plastic waste disposal problem, with the possible added benefit of energy conservation due to methane recovery if biodegradation proceeds by methanogenesis (Young, 1984).

The objectives of this study were to determine the nutritional and incubation conditions that promote anaerobic degradation of PHAs and to obtain microbial populations with the ability to degrade PHAs from different anaerobic environments. A further objective was to isolate and study PHA-degrading microorganisms from these environments. Therefore, the research described in the following sections analyzed each of the terminal electron degradation pathways for PHA biodegradation using concepts and techniques discussed in the literature review.

2. MATERIALS AND METHODS

2.1 Preparation of PHAs Used in Study

P(3HB) and P(3HB-co-3HV) polymers were prepared in shake flask cultures of *A. vinelandii* strain UWD (ATCC 53799) (Page and Knosp, 1989). The medium composition is outlined in Appendix 1.1. Culture volumes of 50 mL in a 500-mL flask were prepared. Cultures were inoculated with a 4% (v/v) inoculum that had been pregrown for 24 h in glucose medium, and incubated at 28 to 30°C under vigorous aeration (225 rpm on a New Brunswick Scientific Co. model G-10 platform shaker) for 20 to 24 h. P(3HB-co-3HV) polymers were formed by the controlled feeding of sodium valerate, that had been neutralized to pH 6.9-7.2 with NaOH, to the glucose- and ammonium acetate-containing cultures during the active P(3HB) production phase (Page *et al.*, 1992). For example, 10 mM sodium valerate was added to cultures at 12 h from the beginning of the incubation period and the cultures were harvested at 21 h in order to obtain a polymer of approximately P(3HB-co-15%3HV). The polymer-containing granules were extracted from the cells with commercial bleach for 60 min at 45°C and purified (Law and Slepecky, 1961). The purification scheme included a distilled water wash to remove cell debris, followed by ethanol and acetone washes to remove lipids. The acetone was allowed to evaporate from the extracted polymers. The resulting insoluble polymer powders were washed with distilled water to remove all traces of acetone and allowed to dry. The polymers were ground in a mortar and pestle to a fine powder and this was used as carbon sources for subsequent experiments. On occasion, solution cast films were prepared. These were made by dissolving either 1% (w/v) or 2% (w/v) dried polymer in chloroform, pouring the solution onto a glass petri plate and allowing the chloroform to evaporate.

2.2 General Anaerobic Culture Techniques

Strict anaerobic techniques were used for all inoculations and incubations of experimental cultures unless described otherwise. Serum bottles of various sizes were used for cultures and prepared using a variation of the Hungate technique (Miller and Wollin, 1974). For methanogenic cultures, medium WR86 (Fedorak and Hrudey, 1984) (see Appendix 1.9) was boiled for 2 min to remove O_2 with subsequent flushing of the medium with a steady flow of 30% CO_2/N_2 gas that had been scrubbed free of O_2 by passage through a heated copper column (Hungate, 1969). The medium was equilibrated to pH 6.9-7.1 with the addition of sodium bicarbonate and maintained in the anaerobic state by continued flushing of the headspace and medium with the O_2-free gas. The medium was transferred with a pipette to serum bottles that had been also purged with the same O_2-free gas via a gas dispersion tube and canula for approximately 2 min. The pipette had been rinsed with the O_2-free gas by withdrawing and expelling gas from the headspace of the medium flask several times. The serum bottles were flushed an additional 30 s with butyl rubber stoppers partially in place. The canulas were then removed while at the same time the stoppers quickly pushed in and seated in order to minimize contact with air. Stoppers were held in place with crimped aluminum caps. The medium was then sterilized by autoclaving (Hungate, 1969).

Media for nitrate-, ferric iron- and sulfate-reducing conditions were basically prepared in the same manner as for the methanogenic cultures. The compositions of the media are given in Appendix 1. The headspace gases were either N_2 or He. The gases were passed through a heated copper column (Sargent Welch) to remove of all traces of oxygen. The pH of the media was equilibrated during the preparation of the media prior to boiling and sparging with O_2-free gas.

Solutions, including carbon sources, reducing agents and inhibitors added after sterilization of the media were prepared anaerobically by the same methods described above. Carbon sources and inhibitors were prepared as stock solutions and boiled and sparged with O_2-free gas. The solutions were then dispersed into serum bottles or Hungate tubes also sparged with O_2-free gas. Reducing agents were added directly to Hungate tubes, sparged with O_2-free gas and boiled O_2-free distilled water was then added to give the desired concentration. Hungate tubes were sealed with Hungate caps as described by the method above. Solutions were sterilized by autoclaving. The solutions were added to serum bottle cultures with sterile syringes and needles that had been rinsed with the appropriate O_2-free gas by withdrawal and expulsion of the gas from a serum bottle that allowed a steady flow of gas into and out of the bottle. For the methanogenic cultures, the sterile medium in the serum bottles was reduced with 2.5% sodium sulfate (prepared anaerobically) just prior to inoculation. PHAs were added to serum bottles in the appropriate amounts and sterilized after the addition of the medium by autoclaving. Stock solutions of carbon substrates were not prereduced.

The inocula were added with sterile syringes and needles that had been rinsed with the appropriate O_2-free gas, unless otherwise indicated. The cultures were set up in triplicate and incubated in the dark. Inoculation volumes, medium volumes, substrate concentrations and incubation temperatures for the various experiments are described in detail before the presentation and discussion of each experiment in the ensuing chapters.

Solid medium for the growth of anaerobic PHA-degrading bacteria (Appendix 1.10) was prepared and allowed to become reduced by placing the agar plates in an anaerobic hood (Coy Laboratory Products Inc., Ann Arbor, Michigan) containing 5% CO_2/10% H_2/85% N_2. All dilutions and streaking of cultures were performed in the anaerobic hood (Section 6.1).

2.3 Isolation, Detection and Identification of Facultative Anaerobic PHA-Degrading Organisms

The ability of isolated facultative anaerobic microorganisms to degrade PHAs under aerobic conditions was routinely verified by plating the organisms onto PHA overlay plates. The bottom agar portion consisted of Burk's medium (Appendix 1.11) with no carbon source. The top agar overlay consisted of the same mineral medium with 3% (w/v) finely powdered PHA granules evenly dispersed (Appendix 1.11). Halos of removed granules around colonies indicated the presence of active depolymerase secretion.

Isolated PHA-degrading organisms were identified using the API 20E System (API Laboratory Products Ltd., St. Laurent, Quebec). The procedure was followed as outlined in the accompanying manual in the kit. Several strains not identified by the above system were sent to the Alberta Environmental Centre in Vegreville, Alberta for identification.

2.4 Analytical Methods

2.4.1 Molecular Weight Determination

Molecular weights of the PHAs were determined by gel permeation chromatography (GPC) using a Shimadzu LC-6A HPLC system containing a Phenogel (10 µm particle size and 10^6 Angstoms pore size) (Phenominex, Torrance, CA) column followed by a Progel-TSK G5000-H6 (10 µL particle size, Supelco) column and a refractive index detector with chloroform (flow rate of 1 mL/min) as the elution solvent. PHA samples were dissolved in chloroform (0.5% w/v) and 50 µL was analyzed within 24 h of sample preparation. Polystyrene molecular weight standards (Aldrich, Milwakee) dissolved in chloroform (0.1% w/v) were used to construct the calibration curve.

2.4.2 PHA Composition Determination

Approximately 2 mg of purified PHA was subjected to methanolysis (Brandl *et al.*, 1988). The PHAs were placed into 13 mm x 100 mm screw-capped tubes. To each tube 1.0 mL chloroform, 0.85 mL methanol and 0.15 mL concentrated sulfuric acid were added. The tubes were then sealed and incubated at 100°C for 140 min, after which the resulting 3-hydroxy-carboxylic acid methyl esters were extracted into chloroform. The 3-hydroxycarboxylic acid methyl esters were separated on a fused silica capillary column (Nukol: 15 m length, 0.53 mm inner diameter, Supelco) housed in a Hewlett Packard (HP) 5890A gas chromatograph. Calculation of methyl ester peak areas was done with a HP 3390A integrator. Retention times of the desired peaks were compared to retention times of commercial PHA products (Aldrich, Milwakee) prepared for GC in the same manner as above.

2.4.3 Methane Analysis

The method of Fedorak and Hrudey (1983) was used to analyze the headspace gases of the cultures for methane. A Microtek GC equipped with a 2 m by 2 mm glass column packed with GP 10% SP1000/1% H_3PO_4 on 100/120 chromosorb WAW (Supelco) was initially used to quantitate methane. N_2 was the carrier gas at 20 mL/min. The flame ionization detector was supplied with 300 mL/min air and 30 mL/min hydrogen. This GC was superseded by a HP model 5700 GC equipped with a flame ionization detector and a 6 ft column packed with Chromosorb 104 (80/100). N_2 was the carrier gas at a flow of 24 mL/min. The injection port, oven and detector temperatures were 25, 25, and 200°C respectively.

Gas tight Lo-dose 1/2 cc u-100 insulin syringes with 28G1/2 needles, (Becton Dickinson, Rutherford , NJ) were used to withdraw 0.1 mL of headspace gases and to inject them onto the GC column. CO_2 (dry ice placed in a sealed flask containing a sampling port) was used to rinse the syringe between samples to prevent O_2 contamination and carry over of methane to the next sample. Quantitative standards were prepared by the addition of known volumes of methane to sealed serum bottles of known volume to give a range of percent methane spanning the amount of methane in the cultures. Peak areas recorded by HP model 3390A integrator were used to determine percent methane in the headspace gas.

2.4.4 Gaseous CO_2, Dissolved CO_2, N_2O and N_2 Analyses

The gases CO_2, N_2O and N_2 in culture headspaces were separated and measured using a Varian Aerograph model 700 GC with a 3 m by 0.5 cm column packed with Poropak R. The GC was fitted with a thermal conductivity detector operated at 25°C and 150 mV. Helium was the carrier gas at 107 mL/min. GC oven and injector temperatures were 60°C and 24°C respectively.

Headspace gases from cultures were sampled as for methane analyses. Syringes were rinsed with CO_2 as described above. This did not interfere with CO_2 detection. Quantitative standards of CO_2 and N_2O were prepared as for methane. A HP model 3390A integrator was used for peak area measurements. When cultures were prepared under a He headspace, N_2 production, in addition to CO_2 and N_2O production, was specifically monitored. Since some N_2 was carried over from the inocula, the test cultures had different amounts of N_2 in their headspaces at the start of incubation. Therefore, 8 mL of CH_4 were injected into cultures at time of inoculation to serve as internal standards and the relative amount of N_2 produced in the cultures was determined by comparing its peak area to the methane peak area. The ratio of N_2 to CH_4 peak area was normalized by dividing each new ratio measurement into the time zero ratio.

To account for any CO_2 dissolved in the culture medium, a modified method of Braun and Gibson (1984) was used. Culture samples of 1 mL were removed with sterile O_2-free syringes and placed into 15 mm x 45 mm, 1 DRAM screw cap vials (Article number 60910L, Kimble, Vineland, NJ) that were capped with a Hungate cap containing a silicone coated liner. Using a syringe, 500 µL of 1.2 M HCl was added to the vial. After 24 h, 500 µL of the headspace gas was analyzed by GC as described above. Quantitative standards of $NaHCO_3$ solutions acidified in the same manner as the culture fluid samples and analyzed in the same manner as the test samples were used to determine the concentration of the released CO_2.

2.4.5 Gas Volume Determination

Gas volumes were determined with a pressure transducer (Micro Switch 142 PC 30G; Honeywell, Freeport, Ill.) (Shelton and Tiedje, 1984). At time of inoculation, cultures were adjusted to ambient atmospheric pressure using a manometer (Fedorak and Hrudey, 1983). The observed CH_4, CO_2 or N_2O concentrations (in percent by volume) were corrected for the presence of water vapor. These values along with the measured volumes from the transducer readings which were corrected for the presence of water vapor and adjusted to atmospheric pressure, allowed a calculation of dry gas amounts (Fedorak and Hrudey, 1983). Calculations were done using the APL program TRANSDRYGAS2 (P.M. Fedorak, University of Alberta, Edmonton) on the Apple Macintosh computer. Calibration standards were prepared on the same day as culture inoculation to eliminate variability of atmospheric pressure. Serum bottles of same size containing equal volumes of water as the cultures, were sealed and equilibrated to ambient atmospheric pressure for 1 h by means of a needle inserted into the stopper to allow for flow of air. Known volumes of CH_4 were injected into the bottles. Standard curves were calculated each time the pressure of the samples were measured.

2.4.6 Nitrate Analysis

Nitrate ions were determined using a HP Anion Chromatography System (Hewlett Packard, Germany) adapted to fit a Waters M-45 (Millipore) HPLC system. A HP ion chromatography column (125 mm by 4 mm) was heated at 40°C in a Shimadzu CTO-6A column oven. The mobile phase was prepared as outlined in the HP Anion Chromatography System user's guide (HP Part No. 90027). A flow rate of 1.5 mL/min was used. The detector was a Waters 486 Tunable Absorbance Detector (Millipore) with signal polarity switching. The detection wavelength was 266 nm and a negative polarity was used. Approximately 0.5 mL of culture fluid was removed for nitrate analysis using a sterile, O_2-free syringe and stored frozen in an Eppendorf tube at -20°C until analysis was done. Before analysis, particles were removed by centrifugation and the samples were diluted either 1 in 50 or 1 in 20 with deionized, distilled, ultrapure water (Milli-Q Reagent Water System, Millipore Continental Water Systems, Millipore Corporation, Bedford, MA). Twenty-five microliters of this solution was then injected onto the column with a Hamilton 50 µL syringe. Standards of KNO_3 were prepared and used to construct calibration curves from peak areas recorded by a HP model 3390A integrator. Calibration curves were constructed each time a new mobile phase was used, the amount of nitrate in the culture supernatant was calculated from these curves.

2.4.7 3-Hydroxybutyrate Determination

The amount of 3HB present in a liquid sample was determined by capillary column GC (Ramsay *et al.*, 1989). Samples of 1 mL were stored in Eppendorf tubes at -20°C until time of analysis. Samples were subjected to methanolysis by heating the samples at 55°C for

45 min in 1 mL methanol (containing 0.5% butyric acid as internal standard) and 200 µL sulfuric acid. The resulting 3-hydroxycarboxylic acid methyl esters were extracted in 1 mL dichloromethane. The methyl esters were separated on the same capillary column used for PHA composition determination. Standards of 3HB were also prepared and analyzed in the same manner as the samples and used to construct calibration curves. 3HB concentrations were calculated from these curves.

2.4.8 Volatile Organic Acids

Volatile organic acids (VOAs) with 2 - 5 carbon atoms in chain length were determined by the method outlined by Roberts (1989). An HP 5700 GC was used with a 2 m by 2 mm glass column packed with GP10% SP1000/1% H_3PO_4 on 100/200 chromosorb WAW (Supelco). N_2 was the carrier gas at 60 mL/min. The flame ionization detector was supplied with 200 mL/min air and 60 mL/min hydrogen. Injector and detector temperatures were 225 and 250°C respectively. Oven temperature was either 120°C or 130°C depending on degree of peak resolution needed (short chain VOAs could successfully be resolved at 120°C, higher chain VOAs at 130°C). Peak areas were obtained using a HP model 3390A integrator. A volume of 100 µL of culture supernatant was sampled and acidified with 10 µL of 4 M phosphoric acid. One µL of this was injected onto the GC column. Quantitative standards of the VOAs were prepared and treated in the same manner as the samples. The standards were used to construct calibration curves which were used to calculate the concentrations of the samples.

2.4.9 Determination of Ferric Iron-Reduction

Ferric iron (FeIII)-reduction was determined by measuring the formation of Fe(II) from the reduction of Fe(III) with a colorimetric assay using 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine or Ferrozine (trademark name) (Sigma Chemical Company, St. Louis, Mo) prepared as a 1.0 g/L solution in 50 mM HEPES (N-2-hydroyethylpiperazine-N'-2-ethane-sulfonic acid, BDH Inc., Toronto). Ferrozine binds with Fe(II) to form a soluble, magenta complex species. The pH was adjusted to 7.0. One mL of the culture fluid was acidified for 15 min at room temperature in 5 mL 0.5 M HCl, after which 0.1 mL of the acidified sample was added to 5 mL of the Ferrozine-HEPES solution and mixed. The absorbance of the sample at 525 nm was measured immediately. The concentration of Fe(II) was calculated from a standard curve prepared in exactly the same method as just described. Standard Fe(II) samples were prepared from ferrous ammonium sulfate to give a 2.0 g FeII/L stock solution. A dilution series of 40, 100, 200, 300, 400, 500 µg Fe(II)/mL was prepared and stored in volumetric flasks at 4°C. Reagent blanks showed no magenta color formation, therefore, no special precautions were taken to prepare iron-free glassware or media.

2.4.10 Chemical Analyses

The proportions of carbon in a P(3HB) and a P(3HB-co-20%3HV) sample were determined by the Microanalytical Laboratory of the Department of Chemistry, University of Alberta. Determination of N_2O in culture headspace gas was done by the Mass Spectrometry Laboratory of Department of Chemistry, University of Alberta.

2.4.11 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed by G. Braybrook in the Department of Geology, University of Alberta. Samples of PHA film were first fixed overnight with 2.5% glutaraldehyde in Milloning's buffer (Milloning's buffer contains per 1.0 L distilled water, 16.8 g $NaH_2PO_4 \cdot H_2O$; 3.86 g NaOH; 5.4 g glucose and 0.05 g $CaCl_2$). The samples were then washed three times for 15 min each with Milloning's buffer and dehydrated through a graded series of 50, 70, 80 and 90% ethanol solutions for 15 min at each gradation with a final dehydration three times for 10 min in 100% ethanol. The samples were then prepared by critical point drying and gold sputtering. The samples were examined in a Cambridge Stereoscan 250 scanning electron microscope at 20 kV.

2.4.12 Statistical Methods

APL functions for the Apple Macintosh computer were used to evaluate data statistically. For simple linear regression analysis, Cricket software for the Apple Macintosh computer was used. The method of Dunnett (1955) was used to evaluate data from a series of test treatments to a control treatment. When comparisons to control cultures were not required, an ANOVA analysis and Duncan's multiple range test was used (Steal and Torrie, 1980). Student's t-test was used for comparing two test treatments. All of the statistical analyses were performed testing the H_o hypothesis at $P<0.05$.

3. BIODEGRADATION OF PHAS UNDER NITRATE-REDUCING CONDITIONS

Microbial denitrification is used to remove nitrate from drinking water polluted by agricultural runoff. Traditionally, a liquid organic substrate is dosed to the water as substrate for the denitrifier to reduce nitrate (Wurmthaler and Müller, 1993). Recent work has concentrated on studying the efficacy of PHAs in serving as substrates and matrices for microorganisms in the denitrification of drinking water (Biedermann *et al.*, 1993). PHAs may, therefore, be biodegradable under these conditions. The objectives of this section were to test various environments for the presence of nitrate-reducing consortia capable of degrading PHAs. Spring water from Whitemud Creek in Edmonton, Alberta; sewage sludge from the second digestor and activated sludge from the fourth pass of the secondary aeration tanks both from the Gold Bar Wastewater Treatment Plant in Edmonton, Alberta were sampled.

3.1 Experimental Procedures

The medium used for all experiments was amended nitrate-reducing medium (Appendix 1.2). Cultures were prepared in triplicate with culture volumes of 50 mL in 158-mL serum bottles. PHAs, P(3HB) and P(3HB-co-15%3HV), were added as dry powders to give a final concentration of 1.0 g/L. The PHAs were added to the serum bottles before the dispensing of the medium. The medium and PHAs were autoclaved to sterilize. The sodium salts of acetate, butyrate and 3HB were added just prior to inoculation from sterile 1 M stock solutions to give a final concentration of 40 mM. No reducing agent or resazurin were added to the cultures. Bromoethanesulfonic acid (BESA) was added from a 1 M sterile solution to give a final concentration of 50 mM. BESA is a known inhibitor of most methanogens (Bouwer and McCarty, 1983). Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) was added from a 1 M sterile solution to give a final concentration of 20 mM. Sodium molybdate is known to inhibit SRB (Banat *et al.*, 1983).

Spring water was inoculated at 10% (v/v) into 50 mL nitrate-reducing medium in 159-mL serum bottles. The carbon substrates tested were the sodium salts of acetate, butyrate and 3HB and P(3HB) and P(3HB-co-15%3HV). As well, cultures amended with yeast extract (0.1% w/v) and unamended cultures were prepared. The cultures were prepared in triplicate and incubated without shaking at room temperature in the dark for 70 days. CO_2 production was routinely measured.

Enrichment cultures of spring water were prepared by inoculating 5 mL spring water into 50 mL Trypticase Yeast Nutrient (TYN) medium (Bossert *et al.*, 1986). TYN is composed of 15 g/L trypticase soy broth, 5 g/L yeast extract and 3 g/L KNO_3 (pH 7.0). The cultures were incubated without shaking in the dark at room temperature for 2 weeks during which time the turbidity of the cultures was observed to slowly increase. A 10% (v/v) inoculum of this enriched spring water culture was used to inoculate nitrate-reducing medium. The same substrates and concentrations as for the previous spring water cultures were used. Unamended cultures were also included. Cultures were prepared in triplicate and incubated without shaking in the dark at room temperature for 69 days.

Sewage sludge was inoculated at 2% (v/v) in 10 mL nitrate-reducing medium in 59-mL serum bottles. The same substrates and concentrations as in the spring water cultures were used. Unamended cultures were also included. All cultures were prepared in triplicate without shaking in the dark at 35°C for 43 days. The experiment was repeated and all conditions were kept the same as in the first experiment. A culture set with 0.1% (w/v) yeast extract and no nitrate was prepared as well. The cultures were incubated without shaking in the dark at 35°C for 26 days.

When activated sludge served as the inoculum, cultures were set up with P(3HB), P(3HB-co-15%3HV), and 3HB as sole carbon and energy sources. Unamended cultures (no carbon substrate added) were also prepared and served as the negative control. The inoculum volume was 10% (v/v). Parallel cultures were set up which contained no added nitrate (the addition of KNO_3 to the amended nitrate-reducing medium was omitted). The cultures were incubated without shaking at room temperature in the dark for up to 92 days. CO_2 production and nitrate reduction were routinely measured.

Nitrate-reducing activated sludge cultures amended with P(3HB) and P(3HB-co-15%3HV) were transferred at 20% (v/v) into corresponding fresh medium prepared under anaerobic conditions containing the PHA at 1.0 g/L. Unamended cultures were transferred into fresh, unamended medium that had also been prepared under anaerobic conditions. The headspace gas was He instead of N_2 as was with all the previous cultures. To ensure a positive pressure in culture headspace for pressure transducer measurements, 10 mL of He was added to each culture. At time of inoculation, 8 mL of methane was added to the headspace gas as an internal standard. The proportion of methane in the headspace gas was expected to remain constant over the entire incubation period. This allowed a comparison of N_2 peak height to methane peak height. Ratios were normalized to time zero readings.

CO_2, N_2O and N_2 production were therefore measured in addition to the reduction of nitrate. As well, the amount of CO_2 partitioned in the culture fluid was measured.

To determine the amount of PHAs left in the cultures at the end of the 40 day incubation period, the cultures were first freeze-dried for 24 h. Once freeze-dried, 50 mL of chloroform was added to each culture and gently shaken overnight to dissolve the PHAs into the chloroform. Particulate matter not soluble in the chloroform was present as well. Two milliliters of each chloroform mixture was added to 13 mm x 100 mm screw-capped tubes and the chloroform allowed to evaporate. The tubes containing the particulate matter and presumably PHA left behind by the evaporated chloroform were subjected to methanolysis and analyzed by GC as described in section 2.4.2. Standards of P(3HB) and P(3HB-co-15%3HV) were prepared by adding known amounts of the polymers to 50 mL of water and freeze-drying the mixtures. The standards were then prepared for GC analysis in the same way as the test samples. A calibration curve constructed from the peak areas of the standards as recorded by the integrator was used to calculate the amount of PHA remaining in the cultures.

3.2 Attempts to Demonstrate PHA Biodegradation by Mixed Cultures of Spring Water and Anaerobic Sewage Sludge Under Nitrate-Reducing Conditions

Preliminary experiments were designed to screen for active populations that could degrade PHAs to CO_2. Thus the activities of the cultures were monitored by measuring CO_2 production. CO_2 production was not detected in spring water cultures as CO_2 levels were less than 0.8% in all of the test cultures after 21 days of incubtion. The inoculation of an enriched spring water culture grown under nitrate-reducing conditions into fresh medium resulted in CO_2 production in all cultures. However, as Table 3.1 shows, only the CO_2 production in 3HB-amended cultures was significantly greater than the CO_2 production in the unamended cultures. The CO_2 production in the PHAs-amended cultures were in fact significantly lower than in the unamended cultures (Table 3.1). The number and diversity of nitrate-reducing bacteria in the spring water may have been very low. The inoculum size (at first 5% v/v and then 10% v/v) may have been too small and resulted in an uneven distribution of bacteria amongst the serum bottle cultures.

Tables 3.2 and 3.3 show the CO_2 production in sewage sludge cultures under nitrate-reducing conditions. In the first experiment (Table 3.2), CO_2 production in acetate-,

Table 3.1 CO_2 production from cultures inoculated with 5 mL of enriched spring water in 50 mL of nitrate-reducing medium after 69 days of incubation at room temperature.

Substrate	Concentration (g/L)	Mean % CO_2 (± std dev)	CO_2 significantly greater than unamended cultures
Unamended	-	5.8 ± 0.12	-
Yeast Extract	0.05	5.9 ± 0.34	no
Acetate	5.4	5.5 ± 0.32	no
Butyrate	3.5	5.5 ± 0.048	no
3HB	5.0	6.7 ± 0.38	yes
P(3HB)	1.0	4.4 ± 0.062	no
P(3HB-co-15%3HV)	1.0	0.59 ± 0.12	no

Table 3.2 CO_2 production from cultures inoculated with 1 mL of sewage sludge in 50 mL of nitrate-reducing medium after 43 days of incubation at 35°C.

Substrate	Concentration (g/L)	Mean % CO_2 (± std dev)	CO_2 significantly greater than unamended cultures
Unamended	-	5.0 ± 0.32	-
Yeast Extract	0.05	4.9 ± 0.48	no
Acetate	5.4	6.5 ± 0.18	yes
Butyrate	3.5	6.0 ± 0.14	yes
3HB	5.0	7.9 ± 0.47	yes
P(3HB)	1.0	4.3 ± 0.27	no
P(3HB-co-15%3HV)	1.0	8.0 ± 0.57	yes

Table 3.3 CO_2 production from cultures inoculated with 1 mL of sewage sludge in 50 mL of nitrate-reducing medium after 26 days of incubation at 35°C.

Substrate	Concentration (g/L)	Mean % CO_2 (± std dev)	CO_2 significantly greater than unamended cultures
Unamended	-	2.0 ± 0.46	-
Yeast Extract	0.05	2.4 ± 0.30	no
Acetate	5.4	2.7 ± 0.53	no
Butyrate	3.5	1.6 ± 0.21	no
3HB	5.0	6.5 ± 1.69	yes
P(3HB)	1.0	1.7 ± 0.25	no
P(3HB-co-15%3HV)	1.0	2.3 ± 0.46	no
Yeast extract no nitrate	0.05	1.9 ± 0.28	no

butyrate-, 3HB- and P(3HB-co-15%3HV)-amended cultures was statistically greater than in the unamended cultures after 43 days. When the experiment was repeated (Table 3.3), only the 3HB-amended cultures had CO_2 production significantly greater than the unamended cultures after 26 days of incubation. In fact the CO_2 production in all of the cultures (Table 3.3) was less than the production in the first experiment (Table 3.2). There was no difference in CO_2 production whether nitrate was present or not (Table 3.3). Sewage sludge is not a typical environment for nitrate-reducing bacteria (Tiedje, 1988), and so, as for the spring water sample, the numbers of nitrate-reducing bacteria present in sewage sludge may have been too low for detection.

3.3 PHA Biodegradation by Activated Sludge Nitrate-Reducing Consortia

3.3.1 Direct Inoculation of Activated Sludge

Activated sludge is known to have a high number of nitrate-reducing bacteria (Tiedje, 1988). Studies on these bacteria have revealed that they are facultative anaerobes and possess the ability to reduce nitrogen oxides when O_2 becomes limiting (Tiedje, 1988). Nitrate-reducing bacteria, therefore, do not require strict anaerobic media or procedures for growth. Cultures were initially set up in strict anaerobic medium, but poor and highly variable growth occurred. After 30 days of incubation, CO_2 production in all of the cultures was below the CO_2 production (3.4 ± 2.2 CO_2) in the unamended cultures. It was likely that the inoculum under anaerobic conditions was unable to generate sufficient energy to synthesize the required denitrifying enzymes (Tiedje, 1988). Subsequent cultures were inoculated into serum bottles containing aerobic medium and anaerobic conditions were then allowed to develop in the sealed bottles.

To ensure that nitrate reduction, and not respiration, was coupled to PHA biodegradation, cultures were incubated initially with no added substrates. The microbial population consumed O_2 using background organic matter found in activated sludge as carbon and energy sources. Once anoxic conditions developed due to total O_2 consumption as indicated by a leveling off of CO_2 production, the test substrates were added. PHAs were, therefore, added to small cuvettes that could be placed within the serum bottles in such a way that the mouths of the cuvettes were above the culture fluid line. At time of substrate addition, the cultures simply had to be tipped to allow the PHA into the culture medium. 3HB was added to the cultures with sterile needles and syringes from sterile stock solutions. Increased

CO_2 production with the concomitant decrease in nitrate after the addition of substrates could then be attributed to nitrate-reduction and not to respiration.

Representative results are shown in Figure 3.1. Substrates were added after 12 days of incubation. Up to that point the CO_2 production in all of the cultures were similar. It seemed that CO_2 production was beginning to level off by day 10 at about 5%. Further incubation, however, showed that CO_2 production in the unamended cultures did not completely stop until after 30 days of incubation at which time the mean CO_2 was 12.3 ± 0.33%.

The addition of 3HB and P(3HB-co-15%3HV) caused an increased CO_2 production over the unamended cultures within approximately 4 days for 3HB-amended cultures (1.6 fold increase) and 6 days for P(3HB-co-15%3HV)-amended cultures (1.3 fold increase). By 48 days of incubation, CO_2 was beginning to level off at 17.6 ± 0.35% for 3HB-amended cultures and at 15.3 ± 0.24% for P(3HB-co-15%3HV)-amended cultures. It was not until 40 days after inoculation that P(3HB)-amended cultures showed increased CO_2 production over the negative control cultures. CO_2 amounts in P(3HB)-amended cultures leveled off after 48 days of incubation at 15.1 ± 0.26%.

Nitrate analyses revealed that by day 22 (10 days after the addition of carbon substrate) the amount of nitrate in the test cultures were significantly lower ($P<0.05$) than the amount in the unamended cultures (Figure 3.2). By day 26, the nitrate had been completely depleted in 3HB-amended cultures and had been reduced from 1.74 ± 0.21 mg/mL to 0.21 ± 0.14 mg/mL in P(3HB-co-15%3HV)-amended cultures at day 30 (Figure 3.2), however, this was not significantly different ($P<0.05$) from the nitrate amounts in P(3HB)-amended and unamended cultures. The addition of a second amount of nitrate resulted in significant nitrate-reduction to occur in P(3HB)- and P(3HB-co-15%3HV)-amended cultures from the unamended cultures. By day 48, the concentration of 3HB in the culture supernatant of 3HB-amended cultures was below 1 mM (1 mg/L).

A second addition of nitrate (3 mg/mL KNO_3) was given to two of the three cultures in each triplicate set at day 50 of the incubation period. This was to see whether an increase in CO_2 production would occur in the nitrate-depleted cultures if nitrate was added. A second addition of 40 mM 3HB was given to two of the three 3HB-amended cultures as well. Results showed an increased CO_2 production in all cases except for the unamended cultures (dashed lines in Figure 3.1). Increases in CO_2 production in PHA-amended cultures given

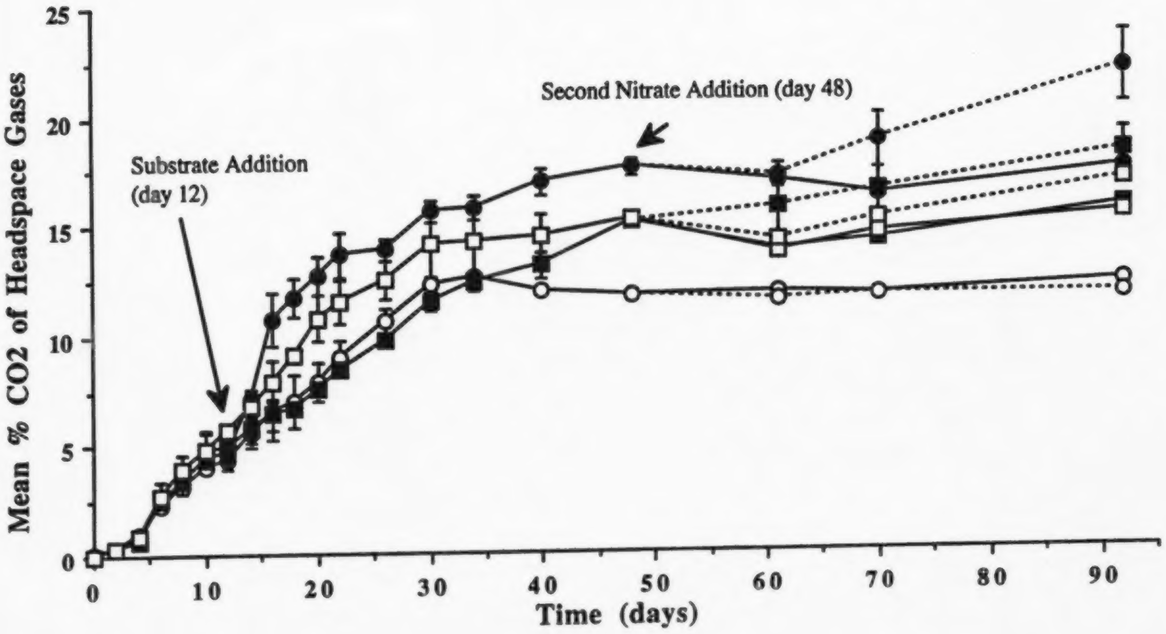


Figure 3.1. Mean cumulative CO_2 production (as % of headspace gases) from activated sludge-containing cultures incubated under nitrate-reducing conditions. -o- unamended; -●- 3HB; -■- P(3HB); -□- P(3HB-co-15%3HV). Dashed lines represent the CO_2 production in two of the three replicate cultures of each test substrate given a second amount of nitrate. Error bars show one standard deviation.

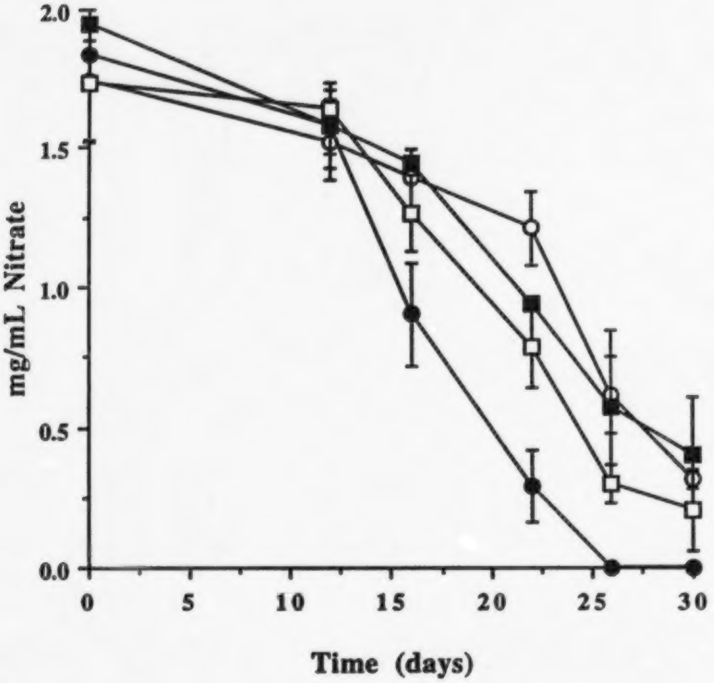


Figure 3.2. Mean nitrate concentrations from activated sludge-containing cultures given nitrate at time of inoculation. -○- unamended; -●- 3HB; -■- P(3HB); -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

extra nitrate and 3HB-amended cultures given extra nitrate and 3HB were between 1.1 to 1.3 fold higher than in their corresponding cultures with no extra nitrate addition or extra carbon substrate as in the case for the 3HB-amended cultures.

Figure 3.3 shows the CO_2 production in cultures not initially given nitrate. Substrates were added at 12 days of incubation. Up to that point CO_2 production in all of the cultures (except P(3HB-co-15%3HV)-amended cultures, which had lower CO_2 amounts) were similar at approximately 7% CO_2 of headspace gases. At time of substrate addition, the amount of CO_2 production was greater (by about 2%) in the cultures without nitrate (Figure 3.3) than in those cultures with nitrate (Figure 3.1). The addition of substrates resulted in no increased CO_2 production in cultures without nitrate (Figure 3.3). The amount of CO_2 in these cultures remained level at approximately 7% for the remainder of the incubation period, and CO_2 production in P(3HB-co-15%3HV)-amended cultures without nitrate was similar to the other cultures from about day 22 onwards.

The addition of nitrate (3 mg/mL KNO_3) at day 27 to two of the three cultures in each culture set resulted in increased CO_2 production over the cultures not given nitrate (Figure 3.3). By day 40, P(3HB-co-15%3HV)-amended cultures given nitrate had significantly higher ($P<0.05$) amounts (1.4 fold) of CO_2 (11.3 ± 1.2% CO_2) than the rest of the cultures and continued to have significantly greater ($P<0.05$) CO_2 production until the end of the incubation period. After 65 days of incubation after given nitrate, the CO_2 in P(3HB)-amended cultures had increased two fold from 7.1 ± 0.04% at day 30 to 14 ± 5.05%, but there were large variations in CO_2 between the two cultures. CO_2 levels in 3HB-amended cultures and unamended control cultures (11.4 ± 0.84 and 10.7 ± 0.26% respectively) were not significantly different ($P<0.05$) 65 days of incubation after given nitrate.

Figure 3.4 shows that at the first nitrate analysis of the culture fluid at day 30 (3 days after the addition of nitrate), there was a significant difference in nitrate concentration. This difference was most likely due to errors arising form analytical techniques. Nitrate concentrations in all cultures either increased then decreased or decreased then increased from day 30 to day 50. Variations in the dilution of the samples for HPLC could have caused these changes in concentration. Nevertheless, the greatest amount of nitrate reduction occurred in 3HB-amended cultures with a decrease of 1.3 mg/mL NO_3^- in 62 days (Figure 3.4). P(3HB-co-15%3HV)-amended cultures had a change of 1.1 mg/mL NO_3^- in 62 days. P(3HB)-amended and unamended cultures had similar amounts of nitrate reduction. By 62 days after the addition of nitrate, the amount of nitrate remaining in the cultures were not significantly

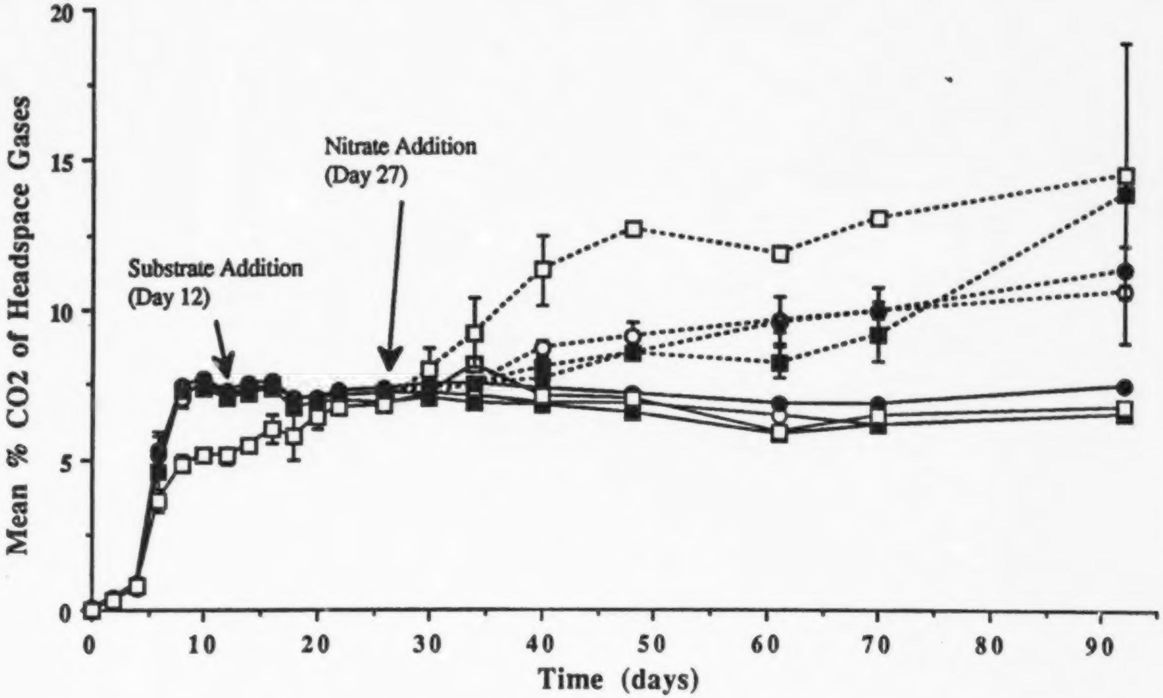


Figure 3.3. Mean cumulative CO_2 production (as % of headspace gases) from activated sludge-containing cultures not initially given nitrate until day 27. On day 27, two of the three cultures were given nitrate and the dashed lines represent the CO_2 production in these cultures. -o- unamended; -●- 3HB; -■- P(3HB); -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

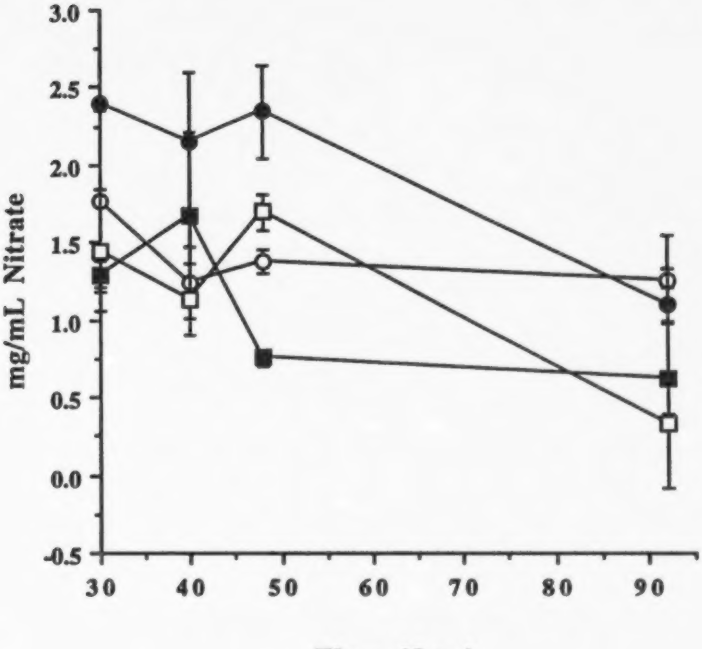


Figure 3.4. Mean nitrate concentrations from activated sludge-containing cultures given nitrate at day 27. -○- unamended; -●- 3HB; -■- P(3HB); -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

different ($P<0.05$). The 3HB concentration in 3HB-amended cultures with or without added nitrate dropped from 40 mM to less than 10 mM within the test period.

The experiments just described were repeated and some different results were obtained. A high degree of variability existed and no significant increases in CO_2 production over the negative control cultures after substrate addition in cultures amended with nitrate occurred. CO_2 production had increased nearly 2-fold in all cultures by the end of the incubation period (66 days). At that time there were significantly greater amounts of CO_2 in P(3HB-co-15%3HV)- and 3HB-amended cultures. A parallel series of cultures were set up without any nitrate. As in the previous experiment, the addition of substrates resulted in no increased CO_2 production yet the addition of nitrate to two of the three cultures did result in increased CO_2 production. Due to the high degree of variability amongst the cultures there were no significant differences in CO_2 production among the cultures given nitrate.

3.3.2 Transfer Cultures of Activated Sludge Nitrate-Reducing Consortia

In order to determine end product formation from the reduction of nitrate, the activated sludge cultures under nitrate-reducing conditions described in the previous experiments were transferred at 20% (v/v) into fresh medium prepared under a He headspace gas. This allowed for the measurements of N_2O and N_2 production in the headspace of the cultures. The cultures transferred were P(3HB)- and P(3HB-co-15%3HV)-amended and unamended cultures.

Figure 3.5 shows the CO_2 production in the transferred cultures. CO_2 production occurred in unamended cultures and this was most likely due to the presence of carbon substrates found in activated sludge that had accompanied the transfers. CO_2 levels increased to $7.3 \pm 0.93\%$ in P(3HB-co-15%3HV)-amended cultures after 40 days. These cultures had significantly greater ($P<0.05$) CO_2 production than the unamended and P(3HB)-amended cultures. CO_2 production in P(3HB)-amended cultures ($4.9 \pm 0.23\%$) was not significantly different ($P<0.05$) from the unamended cultures ($4.7\pm 1.5\%$) after 40 days. The amount of dissolved CO_2 in the culture fluids of P(3HB-co-15%3HV)-, P(3HB)-amended and unamended cultures were 11.0 ± 0.18, 3.5 ± 0.032 and 4.4 ± 0.45 µmol respectively after 40 days. The amount of CO_2 dissolved in the P(3HB-co-15%3HV)-amended cultures was signifcantly greater ($P<0.05$) than in the other two cultures.

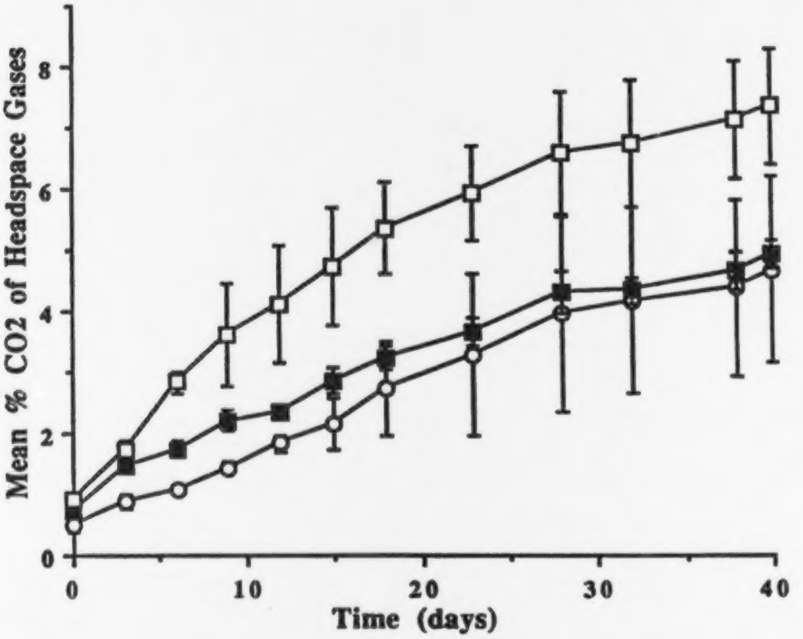


Figure 3.5. Mean cumulative CO_2 production from activated sludge transfer cultures. -o- unamended; -■- P(3HB); -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

Nitrate reduction occurred in all the cultures (Figure 3.6). The greatest amount of reduction occurred in P(3HB-co-15%3HV)-amended cultures. Nitrate decreased from 1.49 mg/mL to 0.09 mg/mL within 40 days in these cultures. Due to large variability amongst the cultures, there were no significant differences in nitrate reduction among the cultures.

Nitrous oxide, N_2O, was shown to be the main N-containing end product of nitrate reduction in all of the cultures (Figure 3.7). The presence of N_2O was confirmed by high resolution mass spectrometry. Since CO_2 and N_2O have approximately the same molecular mass, 10 mL of 10 N NaOH was added to the representative culture to be analyzed. The CO_2 in the gas phase quickly dissolved into the liquid phase under these basic conditions (Fedorak *et al.*, 1982). Analysis of the headspace gases by GC showed that CO_2 had been removed. Therefore, it was ensured that CO_2 would not interfere with the analysis of N_2O by high resolution mass spectrometry. The mass of N_2O was calculated to be 44.00106, the mass of N_2O measured by high resolution mass spectrometry was 44.00130. CO_2 was still detected as a small proportion of the headspace gases. The mass of CO_2 was calculated to be 43.98983, and the measured value was 43.99001.

N_2O production occurred concomitantly with CO_2 production in unamended and P(3HB)-amended cultures during 40 days of incubation (Figure 3.8). The N_2 level remained unchanged in these cultures over the 40-day period (Figure 3.9). The N_2 that was present in these cultures had been carried over with the transfer cultures during inoculation. N_2, however, accumulated in P(3HB-co-15%3HV)-amended cultures after a 10-day lag period (Figure 3.9). The percent N_2O in P(3HB-co-15%3HV)-amended cultures increased to 1.76 ± 0.8 at day 23 and then began to decrease to 1.08 ± 0.91 at day 40 (Figure 3.7). Figure 3.10 shows that both N_2O and N_2 were produced when CO_2 production occurred. However, as CO_2 production increased, the concentration of N_2O decreased while N_2 continued to be produced. This indicated that nitrate was being reduced first to N_2O and then to N_2 within these cultures.

The weights of polymer granules remaining in the PHAs-amended cultures after 40 days were determined by subjecting the granules to methanolysis and measuring the resulting 3-hydroxycarboxylic acid methyl esters by capillary column GC (the method is described in fuller detail in section 3.1 and 2.4.2). The weights of granules remaining in the P(3HB-co-15%3HV)-amended cultures had decreased from 50 mg per culture to approximately 40 mg within 40 days. There was a likewise decrease in P(3HB) granules, from 50 mg per culture

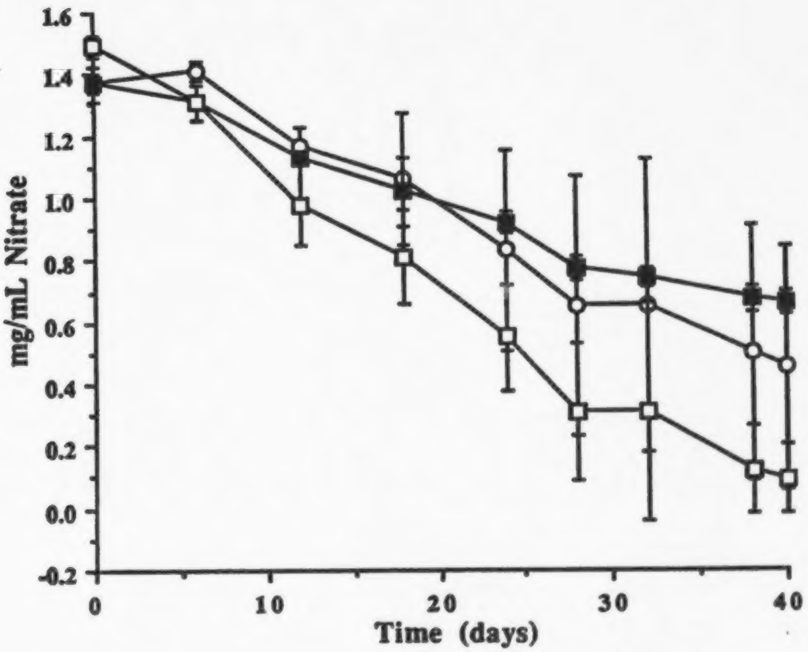


Figure 3.6. Mean nitrate reduction from activated sludge transfer cultures. -o- unamended; -■- P(3HB); -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

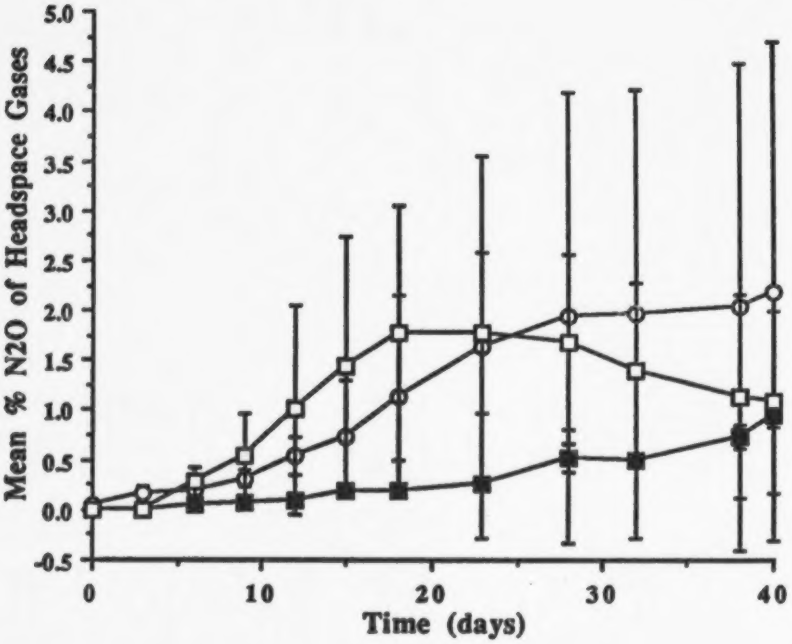


Figure 3.7. Mean nitrous oxide (N_2O) production from activated sludge transfer cultures. -o- unamended; -■- P(3HB); -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

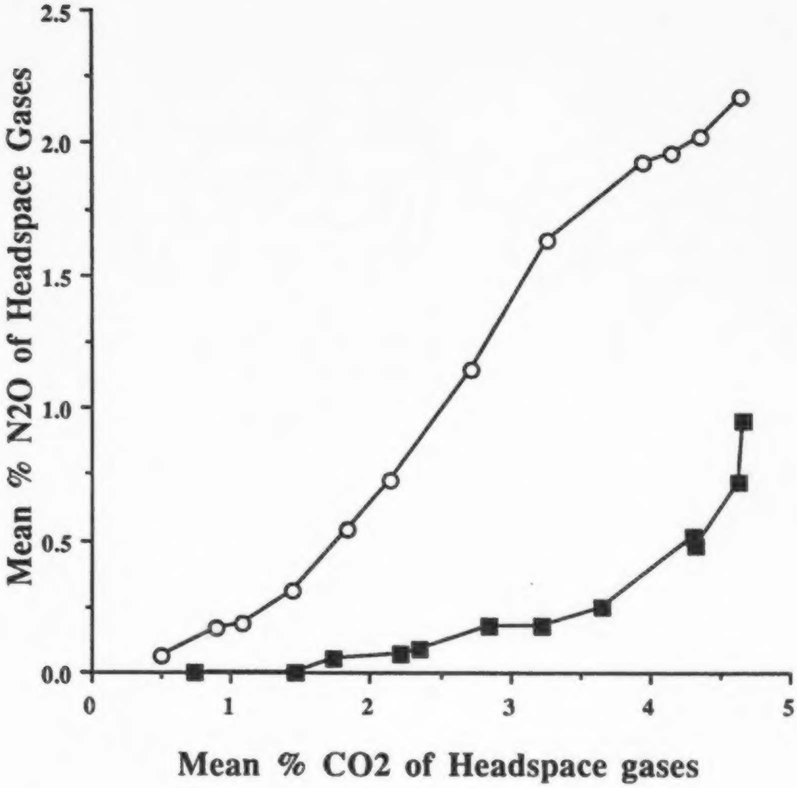


Figure 3.8. Mean % CO_2 and N_2O production during 40 days incubation in unamended (–o–) and P(3HB)-amended cultures (–■–).

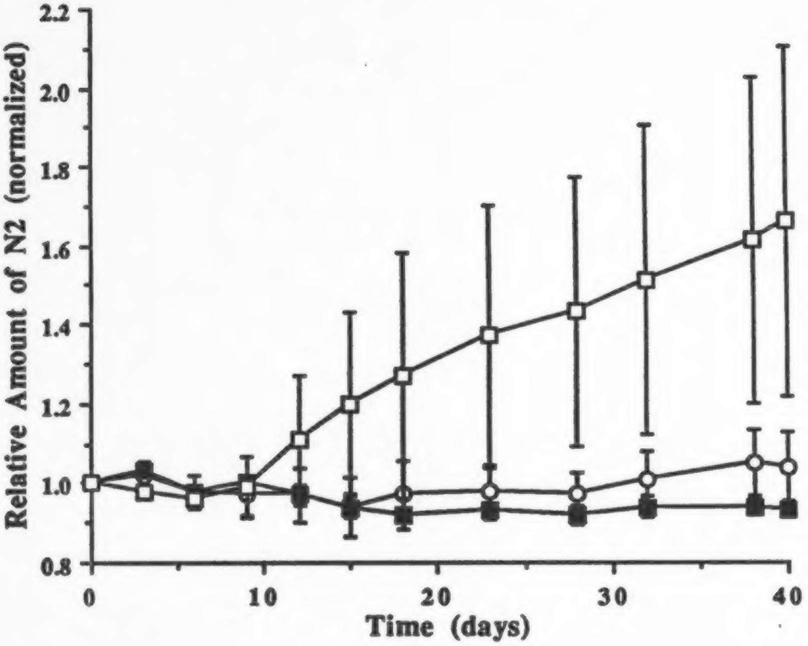


Figure 3.9. Mean N_2 production from activated sludge transfer cultures. -○- unamended; -■- P(3HB); -□- P(3HB-co-15%3HV). Error bars show the standard deviation.

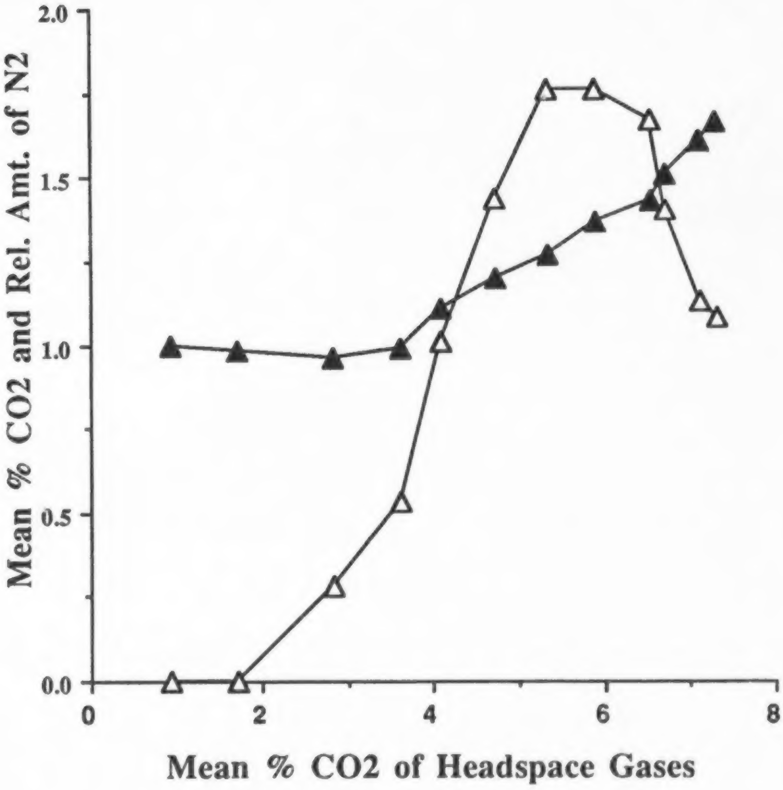


Figure 3.10. Mean % CO_2, N_2O (-Δ-)and N_2 (-▲-) production during 40 days incubation in P(3HB-co-15%3HV)-amended cultures.

initially to about 43 mg per culture at day 40. A visible biofilm could be seen covering the granules, and in most cultures the granules were aggregated together and coated with the biofilm. This clumping of granules and bacterial culture was most pronounced in P(3HB-co-15%3HV)-amended cultures.

3.4 Summary and Conclusions

Cultures growing under nitrate-reducing conditions were obtained when spring water and anaerobic sewage sludge served as inocula only for certain substrates such as 3HB, acetate and butyrate, however, these results were not reproducible. The utilization of PHAs in spring water and sewage sludge cultures under nitrate-reducing conditions could not be demonstrated.

Active nitrate-reducing consortia were obtained with activated sludge as inoculum. Nitrate was shown to be rapidly reduced and depleted within 30 days when cultures were grown at room temperature with 3HB and PHAs as carbon substrates (Figure 3.2). CO_2 production stopped when nitrate became limiting and a burst of CO_2 production was observed when nitrate was added to nitrate-depleted cultures (Figure 3.1). Likewise, increased CO_2 production occurred when nitrate was added to cultures not originally given nitrate (Figure 3.3).

Positive correlations between nitrate-reduction and CO_2 production as well as CO_2 production and N_2O production were demonstrated. Davidson (1991) reports that when the concentration of NO_3^- is high relative to the availability of electron donors (organic carbon substrates), the primary end product of denitrification is N_2O. When NO_3^- concentration is low, N_2 forms as major end product. Nitrate was added at 1.84 mg/mL (30 mM KNO_3) and 3HB at 40 mM and PHAs at 1.0 mg/mL. Results showed that this combination of nitrate and substrate amounts resulted in N_2O end product formation.

The N_2O levels in P(3HB-co-15%3HV)-amended cultures were observed to increase then decrease with time. The correlation between N_2 and CO_2 production increased with time. This shows that these cultures were very actively degrading the polymer and reducing nitrate to N_2O and then to N_2. After 40 days of incubation, the nitrate was nearly depleted in the P(3HB-co-15%3HV)-amended cultures (Figure 3.6), N_2O levels had decreased to about 1% of the headspace gases (Figure 3.7) and the relative N_2 production was approximately 1.6 greater than in P(3HB)-amended cultures (Figure 3.9). In contrast, after 40 days of incubation, there was still approximately 0.7 mg/mL nitrate in the culture fluid of P(3HB)-amended cultures (Figure 3.6), N_2O production had slowly increased to 1% of the headspace gases (Figure 3.7) and the relative N_2 production had remained unchanged (Figure 3.9). The difference in gas production and nitrate reduction between P(3HB-co-15%3HV)- and P(3HB)-amended cultures may be due to the fact that the P(3HB) granules were more crystalline than P(3HB-co-15%3HV) granules thus retarding enzymatic hydrolysis of the ester bonds (Nishida and Tokiwa, 1993). 3HB did not accumulate in the supernatant of any of the polymer-containing cultures. Figure 3.1 shows that 3HB was rapidly converted to CO_2. There was a corresponding rapid reduction in nitrate in 3HB-amended cultures (Figure 3.2). This signifies that 3HB was rapidly metabolized under nitrate-reducing conditions and, therefore, any 3HB units released from the biodegradation of the polymers were readily absorbed by the bacteria.

The reproducibility of the experimental results was poor. High degrees of variability and different results were observed. Considerable variation between replicates were also observed in sediment samples taken by Edwards (1994). She screened sediments from two contaminated sites for the presence of microorganisms capable of biodegrading benzene under anaerobic conditions. Microcosms were set up under nitrate-, ferric iron- and sulfate-reducing conditions as well as under methanogenic conditions. She suggested that despite careful mixing of sediments before dispensing into serum bottles, the microorganisms of interest were not uniformly distributed in the sediment but were present only in small micro-environments. Nitrate-reducing bacteria in activated sludge may also exist in small micro-environments attached to organic matter. To reduce variability larger sample volumes would have to be used but the volumes probably needed would likely be unwieldy and impractical. Another factor contributing to the variability between replicates could be that the organic matter composition of the activated sludge inoculum and its bacterial flora could have changed from one sampling time to the other. It had rained for several days at one sampling time, and at the other sampling time it had been warm and sunny. Despite the unreproducible results, general trends were observed. Namely, with the addition of nitrate to the cultures, the substrates were used faster than if no nitrate were added. N_2O accumulated as the main N-containing end product of nitrate reduction. PHAs have been shown to be biodegradable in activated sludge under aerobic conditions (Briese *et al.*, 1994; Gilmore *et al.*, 1993). As well Shirmir *et al.* (1993) and Tanio *et al.* (1982) isolated PHA-degrading organisms from activated sludge. The results presented in this chapter demonstrated that PHAs, especially P(3HB-co-15%3HV), were biodegraded in activated sludge under nitrate-reducing conditions.

4. ATTEMPTS TO DEMONSTRATE PHA BIODEGRADATION UNDER SULFATE- AND FERRIC IRON-REDUCING CONDITIONS

The purpose of this section was to determine whether PHA biodegradation could occur under sulfate- and ferric iron-reducing conditions using pure cultures and environmental consortia of SRB and ferric iron-reducing bacteria . Environments tested were spring water from Whitemud Creek in Edmonton, Alberta and sewage sludge from the second anaerobic digestor at the Gold Bar Wastewater Treatment Plant in Edmonton, Alberta.

4.1 PHA Biodegradation Under Sulfate-Reducing Conditions

4.1.1 Experimental Procedures for Biodegradation Studies Under Sulfate-Reducing Conditions

Several SRB have been reported to synthesize and store PHA internally (Steinbüchel, 1991). Of these, three were tested for the ability to degrade exogenous P(3HB) and P(3HB-co-15%3HV). These included *Desulfovibrio sapovorans* DSM 2055, *Desulfococcus multivorans* DSM 2059 and *Desulfosarcina variabilis* DSM 2060. The media used and their preparation are outlined in Appendix 1.3. The type of medium and preferred carbon source required for *Desulfovibrio sapovorans* was freshwater and sodium lactate at 2.5 mL/L of a 60% syrup; for *Desulfococcus multivorans*, brackish and sodium benzoate at 0.5 g/L and for *Desulfosarcina variabilis*, marine and sodium benzoate at 0.5 g/L respectively. Cultures were grown with their preferred carbon source for 3 weeks at 35°C until turbid growth was visible. These cultures served as the inocula, with an inoculum volume of 9% (v/v) for *Desulfovibrio sapovorans*, 8% (v/v) for *Desulfococcus multivorans* and 9% (v/v) for *Desulfosarcina variabilis*. The carbon sources tested were P(3HB) and P(3HB-co-15%3HV) at 500 mg/L as sole carbon and energy source. As well, cultures with their preferred carbon substrate and 500 mg/L P(3HB) were set up. Incubation of cultures with the preferred carbon source as sole carbon source were used as positive controls. Cultures were prepared in triplicate with culture volumes of 75 mL in 100-mL serum bottles and incubated without shaking at 35°C in the dark for 130 days. CO_2 production was measured as outlined in section 2.4.4 and used as an indication of bacterial metabolic activity.

After 61 days, *Desulfovibrio sapovorans* and *Desulfococcus multivorans* cultures containing the preferred carbon substrates as sole carbon and energy sources were transferred into fresh medium containing the corresponding preferred carbon source at the same initial concentrations as in the original cultures. Likewise, *Desulfovibrio sapovorans* and *Desulfococcus multivorans* cultures containing the preferred carbon substrates and P(3HB) were transferred into fresh medium containing the preferred carbon source at half the concentration given to the original cultures and P(3HB) at 500 mg/L. The original cultures were transferred at a volume of 13% (v/v) into triplicate 100-mL serum bottles containing 75 mL of the appropriate media, prepared as outlined in Appendix 1.3. The cultures were incubated in the dark at 35°C without shaking for 62 days.

Postgate C medium (Appendix 1.4) was used for the cultivation of spring water cultures. Cultures were prepared in triplicate and final culture volumes were 50 mL in 158-mL serum bottles. PHAs were provided at 1.0 g/L as dry powders. They were added to serum bottles prior to addition of medium and were then autoclaved in the medium to sterilize. The sodium salts of acetate, butyrate and 3HB were added as sterile solutions to give a final concentration of 40 mM. Cultures given 0.1% (w/v) yeast extract were set up as were unamended cultures. Sodium sulfate at a final concentration of 20 mM served as the terminal electron acceptor. Spring water was added at 5% (v/v). Cultures were incubated at room temperature in the dark without shaking for 81 days and CO_2 production measured.

Enrichment cultures were set up by inoculating spring water at 20% (v/v) into Postgate C medium (Appendix 1.4) containing either yeast extract (0.1% w/v) and sodium lactate (2.5 mg/L of 60% syrup) or yeast extract, sodium lactate and P(3HB) or P(3HB-co-15%3HV) at 500 mg/L. The cultures were incubated at room temperature for 3.5 weeks in the dark at room temperture. Cultures amended with yeast extract and sodium lactate served as the inoculum for acetate-, butyrate- and 3HB-amended cultures whereas the cultures grown with yeast extract, sodium lactate and either P(3HB)- and P(3HB-co-15%3HV)- served as the inoculum for P(3HB) and P(3HB-co-15%HV)-amended cultures. The presence of the PHA granules in the inoculum was to select for polymer-degrading bacteria. The inoculum volume was 10% (v/v). The cultures were incubated without shaking at room temperature in the dark for 69 days.

Active spring water cultures were transferred after 4 months into corresponding new medium (Postgate C, Appendix 1.4) that had been prereduced with 2.5% Na_2S

(added at 1% v/v). Cultures were prepared in triplicate. An additional parallel culture was prepared that was not reduced. Culture volumes were 50 mL in 158-mL serum bottles. Inocula volumes were 10% (v/v). The substrates tested included the sodium salts of butyrate and 3HB at 40 mM each and P(3HB) and P(3HB-co-15%3HV) at 1.0 g/L. As well, triplicate cultures amended with sodium lactate (2.5 mg/L of 60% syrup) with and without sodium sulfate were prepared. Unamended cultures were also set up. Cultures were incubated without shaking at room temperature for 41 days.

The same medium and substrate concentrations used in the spring water cultures were used for experiments with anaerobic sewage sludge as inoculum. Cultures were prepared in triplicate in 59-mL serum bottles at a final culture volume of 10 mL. BESA was added to give a final concentration of 50 mM in order to inhibit methanogenesis. A 2% (v/v) inoculum volume was used. Cultures were incubated at 35°C in the dark without shaking for 80 days and CO_2 production measured.

4.1.2 Pure Culture Biodegradation Studies Using SRB

Of the three SRB isolates tested, only *Desulfosarcina variabilis* grew very slowly and poorly and hence was not used in the subsequent studies. A comparison of CO_2 production in *Desulfovibrio sapovorans* and *Desulfococcus multivorans* cultures incubated with the preferred carbon source or with the preferred carbon source and P(3HB) revealed no significant differences ($P<0.05$) existed between the cultures after 61 days of incubation. The percent CO_2 measured in these cultures (preferred carbon-amended and preferred carbon- and P(3HB)-amended respectively) after 61 days ranged from 8.1 ± 0.72 to 9.2 ± 2.4 for *Desulfovibrio sapovorans* cultures and from 10.6 ± 2.3 to 9.2 ± 0.92 for *Desulfococcus multivorans* cultures. In contrast, the CO_2 production in cultures given only PHA was very low and after 61 days the percent CO_2 in these cultures for both strains was below 2. After 130 days of incubation the CO_2 levels in PHA-amended cultures for both strains were below 3%. It could not be distinguished from this experiment whether the SRB cultures were capable of using PHAs as a growth substrate or if the PHA-amended cultures were growing on any preferred carbon substrate carried over in the inoculum.

In order to determine whether those cultures given P(3HB) in addition to the preferred carbon source were actually using P(3HB), the cultures were transferred after 61 days into fresh medium containing P(3HB) granules at 500 mg/L and the preferred carbon source at half the original concentration so that the cultures would use up the preferred carbon source quickly and be forced to use the P(3HB). Figure 4.1 shows the mean percent CO_2 in headspace gases in *Desulfovibrio sapovorans* and *Desulfococcus multivorans* cultures given the preferred carbon source (sodium lactate and sodium benzoate, respectively) and preferred carbon source and P(3HB) after 15 days of incubation at 35°C. CO_2 present in cultures at time of inoculation came from the CO_2 present in the inocula. After 6 days, CO_2 production leveled off in sodium lactate-amended *Desulfovibrio sapovorans* cultures at 12.2 ± 0.45% CO_2. Cultures amended with sodium lactate and P(3HB) had 8.1 ± 0.38% CO_2 in the headspace after 6 days. This was a 1.5 fold difference. After 62 days of incubation this difference in CO_2 production remained the same. Likewise, CO_2 production in *Desulfococcus multivorans* sodium benzoate-amended cultures leveled off after 8 days at 13.1 ± 0.7% CO_2. The cultures amended with sodium benzoate and P(3HB) had 6.1 ± 1.02% CO_2 in the headspace afer 8 days. This was a 2-fold difference. Again, this difference in CO_2 production did not change after 62 days. These results show that the two strains of SRB tested, *Desulfovibrio sapovorans* and *Desulfococcus multivorans*, could not utilize P(3HB) as a carbon or energy source since the amounts of CO_2 produced in the cultures could be accounted for by the catabolism of the preferred carbon substrate. CO_2 production was approximately half in cultures given half the amount of preferred carbon substrate. If the cultures were able to also metabolize P(3HB) the CO_2 production would have been higher.

4.1.3 Mixed Culture Biodegradation Studies Under Sulfate-Reducing Conditions

Spring water was sampled and tested to determine whether SRB were present and could be cultivated and whether a consortium could be established with sulfate serving as the terminal electron acceptor and acetate, butyrate, 3HB and PHAs serving as electron donors. CO_2 production was measured as an indication of metabolic activity.

After 81 days, the highest CO_2 production occurred in 3HB- and butyrate-amended cultures and in unamended cultures with 7.0 ± 0.17, 5.4 ± 3.37 and 5.7 ± 3.29% CO_2 detected in these cultures respectively. Cultures amended with yeast extract had produced 3.0 ± 0.25% CO_2 after 81 days. The percent CO_2 detected in acetate-, P(3HB)- and P(3HB-co-15%3HV)-amended cultures were all below 2 after 81 days.

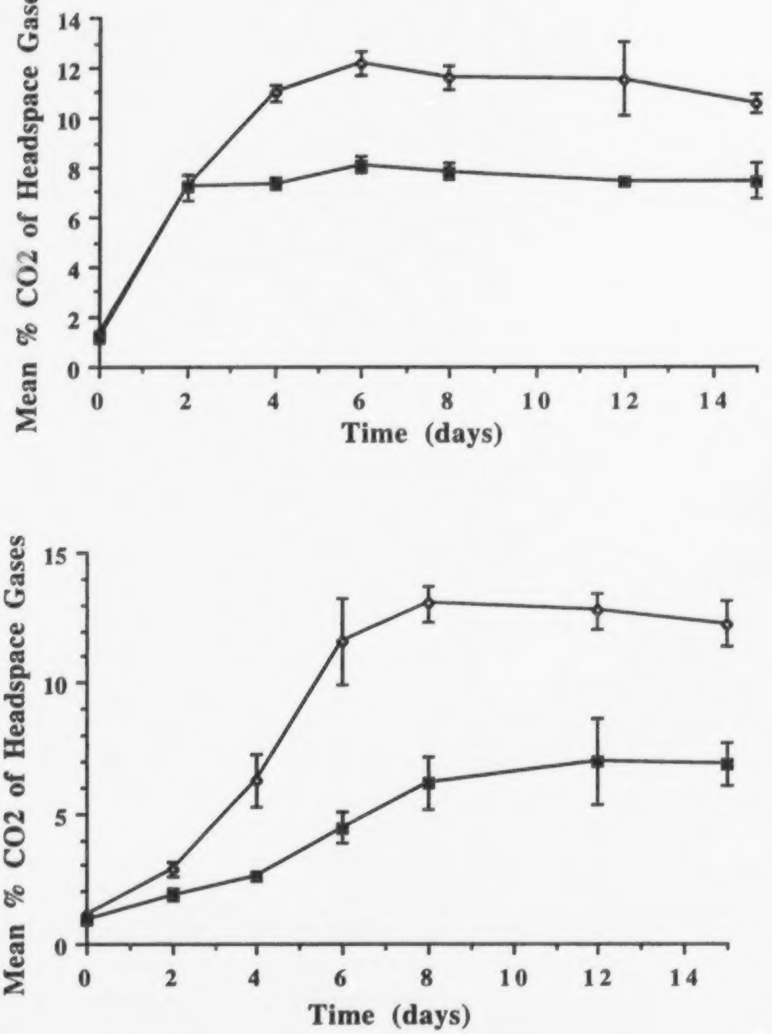


Figure 4.1. Mean cumulative CO_2 production from pure SRB cultures amended with preferred carbon source or the preferred carbon source and P(3HB). a) *Desulfovibrio sapovorans*, -◇- sodium lactate (2.5 mg/L); -■- sodium lactate (1.25 mg/L) and P(3HB) (500 mg/L); b) *Desulfococcus multivorans*, -◇- sodium benzoate (0.5 g/L); -■- sodium benzoate (0.25 g/L) and P(3HB) (500 mg/L). Error bars show one standard deviation.

The unamended cultures served as the negative control. Any CO_2 production in these cultures could be attributed to metabolism of carbon substrates that accompanied the inoculum. It was expected that all the cultures would have at least the same amount of CO_2 production as the unamended cultures due to background carbon substrates. However, the results showed that CO_2 production was highly variable with some cultures having lower CO_2 levels than the unamended cultures. The low inoculum volume (5% v/v) probably caused an uneven distribution of SRB and other bacteria in all of the serum bottle cultures resulting in varied CO_2 production.

In order to obtain an active sulfate-reducing culture from spring water and to lower the variability in CO_2 production, an enrichment step was done. Figure 4.2 shows the mean cumulative CO_2 production in P(3HB)- and P(3HB-co-15%3HV)-amended cultures and unamended cultures after 69 days of incubation. Statistical analysis showed that CO_2 production in P(3HB)- and P(3HB-co-15%3HV)-amended cultures were not significantly different from each other but significantly greater than the CO_2 production in the unamended and other test cultures. The majority of the CO_2 production occurred within the first 8 days of incubation in these cultures, with CO_2 increasing slightly to 3.0 ± 1.3 and 2.3 ± 0.6% after 69 days in P(3HB)- and P(3HB-co-15%3HV)-amended cultures, respectively. CO_2 production in 3HB-, butyrate-, acetate-amended and unamended cultures was below 1.4% CO_2 after 69 days. The majority of the CO_2 production in these cultures occurred within the first 8 days as well.

The error bars (representing one standard deviation) in Figure 4.2 show the high degree of variability in CO_2 production in these cultures. Despite the variability it can be concluded that cultures amended with PHAs were able to utilize these polymers as sole carbon and energy sources due to enhanced CO_2 production. However, it was not determined whether sulfate was being reduced in these cultures.

The previous cultures were not prereduced and it was expected that from the metabolic activities of the microorganisms found in the spring water inoculum, the oxidation-reduction (redox) potential (E_h) of the medium would reduce sufficiently to produce anaerobic conditions. New cultures were set up that were prereduced with Na_2S and contained a redox indicator dye (resazurin) to ensure anaerobic conditions. Parallel cultures containing no reducing agent were also set up. The cultures inoculated directly with spring water and incubated for 81 days served as the inocula. Only 0.04 to 0.3% CO_2 was detected in prereduced butyrate-, 3HB-, P(3HB)- and P(3HB-co-15%3HV)-

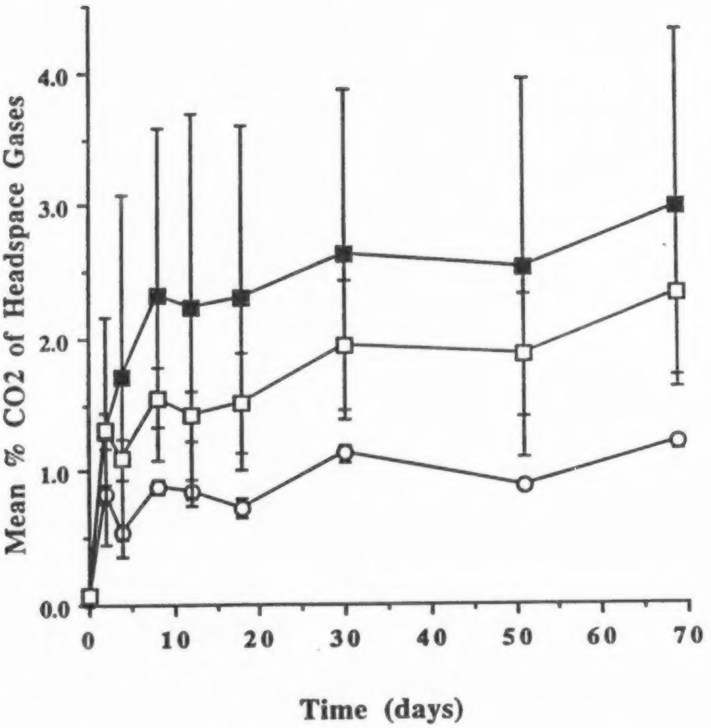


Figure 4.2. Mean cumulative CO_2 production from enriched spring water-containing cultures under sulfate-reducing conditions. -o- unamended; -■- P(3HB) and -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

amended cultures after 41 days of incubation at room temperature. Corresponding cultures with no reducing agent added had CO_2 levels between 1.1 to 1.8% after 41 days. In contrast, cultures amended with sodium lactate as sole carbon and energy source produced 6.0±3.9% CO_2 after 41 days. Cultures amended with sodium lactate but with no added sulfate as terminal electron acceptor produced 2.0±.61% CO_2 after 41 days.

The results showed that cultures grew slowly, if at all, when prereduced. Higher CO_2 production was achieved in cultures when not prereduced. Growth occurred and CO_2 production was higher in cultures amended with sodium lactate and with sulfate present as terminal electron acceptor than when sulfate was not present. The CO_2 production in sodium lactate-amended cultures with sulfate, however, was variable.

Poor activity in terms of CO_2 production (less than 2% CO_2 after 60 days) was observed in the anaerobic sewage sludge cultures amended with PHA, acetate, butyrate and 3HB. The cultures amended with yeast extract produced high levels of CO_2 (9.1±3.1% CO_2 after 80 days) but there was a large amount of variability. From enumeration data of SRB using lactate medium, 4.9×10^5 SRB would be expected in 1 mL of sewage (Fedorak *et al.*, 1987). Therefore, about 9.8×10^4 SRB were added to each serum bottle culture in the experiment when a 2% (v/v) sewage sludge inoculum was used. Other types of SRB, eg. acetate- and butyrate-utilizers, would likely be present in lower numbers and may have been unevenly distributed amongst the cultures.

4.1.4 Conclusions

The results from the spring water and anaerobic sewage sludge experiments showed that it was possible to obtain active cultures under sulfate-reducing conditions. However, the substrate range of the cultures seemed to be limited. Best growth and CO_2 production occurred when yeast extract and/or sodium lactate were present. Yeast extract is a rich source of carbon and nutrients and sodium lactate is a preferred carbon substrate of the sulfate-reducing bacterium, *Desulfovibrio sapovorans*. PHA utilization was demonstrated in one experiment but these results were not reproducible. As well, CO_2 production was highly variable even when enriched, active cultures were used as inoculum. This is probably due to uneven distribution of bacteria due to low inoculation volumes. The inoculation volumes were kept low in order to minimize background bacterial activity.

The SRB strains (*Desulfovibrio sapovorans* and *Desulfococcus multivorans*) were shown not to be able to grow on PHAs. It was not tested whether the SRB strains could utilize 3HB, the monomer of P(3HB). 3HB was shown not to be readily utilized by mixed cultures under sulfate-reducing conditions.

4.2 PHA Biodegradation Under Ferric Iron-Reducing Conditions

4.2.1 Experimental Procedures for Biodegradation Studies Under Ferric Iron-Reducing Conditions

Shewanella putrefaciens was grown on B10 agar plates (B10 medium with 1.8% (w/v) agar) from glycerol stocks (see Appendix 1.5 for composition of B10 medium). An isolated colony was inoculated into a test tube containing B10, capped and incubated at 28°C for 2 days until growth was visible on the bottom of the tube. Sterile syringe and needle were used to inoculate the culture into prepared serum bottles at a volume of 5% (v/v). When B10 medium (Appendix 1.5) was used, PHAs, P(3HB) and P(3HB-co-15%3HV), were added as dry powders at 1.0 g/L and acetate, butyrate and 3HB (as sodium salts) were added from sterile stock solutions to a final concentration of 30 mM. As well, cultures in B10 medium with yeast extract at 0.1% (w/v) with and without Fe(III) as terminal electron acceptor were set up. Uninoculated cultures with Fe(III) were incubated with the test cultures in order to test whether nonenzymatic iron reduction occurred. All cultures were set up in triplicate and final culture volumes were 10 mL in 59-mL serum bottles. Cultures were incubated without shaking at 28°C in the dark for 15 days. CO_2 and Fe(II) production were routinely measured. When modified anaerobic citrate medium (Appendix 1.6) was used, the PHAs were provided at 500 mg/L and the sodium salts of lactate, pyruvate, succinate, acetate, butyrate and 3HB were added from sterile stock solutions to a final concentration of 15 mM. As well, unamended and heat killed (sterile) cultures were set up. All cultures were prepared in duplicate at a culture volume of 50 mL in a 158-mL serum bottle. Cultures were incubated without shaking at 28°C in the dark for 12 days. CO_2 and Fe(II) production were routinely measured.

The medium used for *Geobacter metallireducens* was anaerobic citrate medium (Appendix 1.7). PHAs, P(3HB) and P(3HB-co-15%3HV), were added as dry powders at a concentration of 500 mg/L and the sodium salts of acetate, butyrate and 3HB were added from sterile stock solutions to a final concentration of 50 mM. The inoculum was 10% (v/v) of *G. metallireducens* cultures grown on acetate for 2 weeks at 28°C. Cultures were prepared in triplicate and final culture volumes were 50 mL in 158-mL serum bottles. Incubation was carried out at 28°C in the dark without shaking for 17 days and CO_2 and Fe(II) production routinely measured.

G. metallireducens cultures with strips of Biopol film (1 cm x 7 cm) and P(3HB) solvent-cast film (1 cm x 3 cm) as sole carbon and energy sources were prepared in duplicate as 50 mL cultures in 158-mL serum bottles under a N_2 headspace. A 10% (v/v) inoculum of *G. metallireducens* culture grown on 50 mM sodium acetate for 2 weeks was used. The cultures were incubated without shaking at 28°C in the dark for 6 months.

The ferric iron source used for both spring water and sewage water samples was amorphic Fe(III)oxyhydroxide, $Fe(OH)_3$. A new batch of $Fe(OH)_3$ was prepared each time new cultures were set up (Lovley and Phillips, 1986). A 0.4 M solution of $FeCl_3 \cdot 6H_2O$ was made in distilled water and neutralized to pH 7.0 with NaOH. The resulting slurry was washed with large amounts of distilled water to remove chloride ions. A spot test for the detection of chloride ions involved adding a few drops of 8.5% $AgNO_3$ in distilled water to a small sample of the slurry suspension. Formation of a white precipitate indicated that chloride ions were still present.

Enrichment cultures of a mixture of spring water and spring water sediment were prepared by incubating a 10% (v/v) inoculum in B10 medium amended with yeast extract (0.1% w/v) at room temperature for several days until turbid growth was visible. Enrichment cultures with both yeast extract and P(3HB) or P(3HB-co-15%3HV) were also set up and served as the inocula for cultures amended with P(3HB) and P(3HB-co-15%3HV). The $Fe(OH)_3$ amount added was 250 mmol Fe(III)/L. The cultures were incubated at room temperature for 43 days.

Iron enrichment medium (Appendix 1.8) was prepared for both spring water and sewage sludge cultures. Cultures were prepared in triplicate and final culture volumes were 50 ml in 158-mL serum bottles. $Fe(OH)_3$ was added to the bottles (250 mmol/L) and flushed with N_2 gas for several minutes and then sealed. Medium and carbon substrates were then added next (PHAs were added at time of $Fe(OH)_3$ addition). PHAs, P(3HB) and P(3HB-co-15%3HV), were added at 1.0 g/L for spring water cultures and at 500 mg/L for sewage sludge cultures. The sodium salts of acetate, butyrate and 3HB were added from sterile stock solutions to a final concentration of 40 mM. Inoculum volume of spring water was 5% (v/v) and the cultures were incubated at room temperature in the dark. Sewage sludge cultures were inoculated at 2% (v/v) and incubated at 35°C in the dark. BESA and sodium molybdate were added to the sewage sludge cultures to a final concentration of 50 mM and 20 mM respectively. CO_2 and Fe(II) production were routinely measured.

4.2.2 Pure Culture Biodegradation Studies Using Ferric Iron-Reducing Bacteria

Shewanella putrefaciens ATCC 8071 is a nonfermenting, facultative anaerobic bacteria that can use ferric iron as a terminal electron acceptor (DiChristina and DeLong, 1994). An experiment was conducted to see whether this organism was capable of utilizing PHAs as carbon sources under iron-reducing conditions. Results, however, showed that *S. putrefaciens* could not grow on PHAs, 3HB, acetate or butyrate as CO_2 levels in these cultures were below 1% after 15 days. Iron reduction did occur but only on a limited scale and may be a result of abiotic reduction (Lovley and Phillips, 1988). *S. putrefaciens* grew very well on lactate and pyruvate. After 12 days of incubation, these cultures had produced 3.8 ± 0.45 and 8.6 ± 0.07 % CO_2 on lactate and pyruvate, respectively. These amounts were significantly greater ($P<0.05$) than the CO_2 production in acetate-, butyrate-,3HB-, succinate- and PHAs-amended cultures. There was a corresponding reduction of Fe(III) in the lactate- and pyruvate-amended cultures. After 12 days, the amounts of Fe(II) accumulated in the culture fluid of these cultures were 2.1 ± 0.13 and 2.0 ± 0.12 mg Fe(II)/mL respectively, and these amounts were signifcantly greater ($P<0.05$) than in the other cultures. Therefore, *S. putrefaciens* was shown to be able to grow and reduce Fe(III) on lactate and pyruvate. PHAs, 3HB, acetate and butyrate were not shown to be utilized by *S. putrefaciens.*

Geobacter metallireducens ATCC 53774, recently characterized as a Gram negative-, strict anaerobic bacterium capable of oxidizing several short chain fatty acids, alcohols and monoaromatic compounds with Fe(II) as sole electron acceptor (Lovley *et al.*, 1993), was tested for its ability to utilize PHAs and 3HB as sole carbon sources.

No differences in CO_2 production amongst the culture sets were observed and after 17 days of incubation, 4.0 to 6.0% CO_2 was measured in all of the culture headspaces (Figure 4.3). Fe(II) had been reduced at equal rates and amounts in all

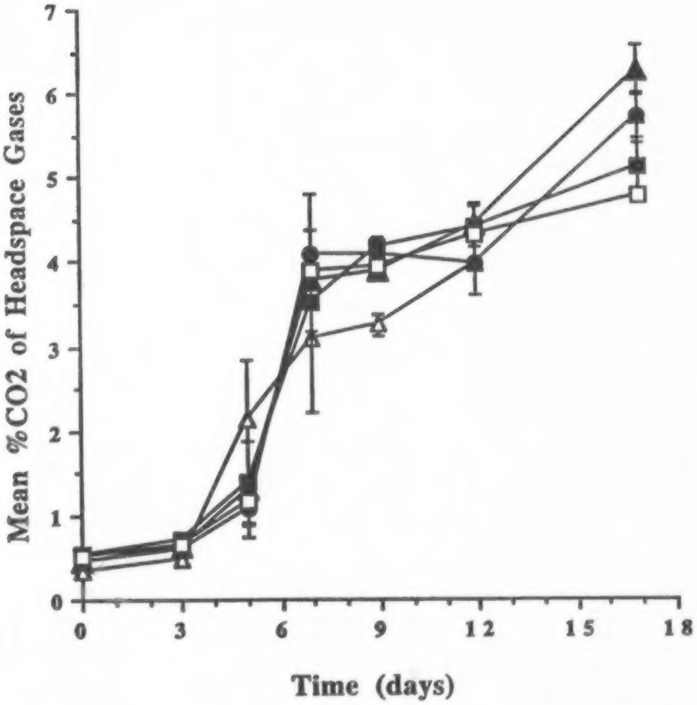


Figure 4.3. Mean cumulative CO_2 production (as % of headspace gases) from *G. metallireducens* cultures. Cultures were amended with: -▲- acetate, -△- butyrate, -●- 3HB, -■- P(3HB) and -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

cultures. At 17 days of incubation, approximately 2.1 mg/mL Fe(II) was measured in each culture. The greatest rate of increase in CO_2 and Fe(II) production occurred from day 3 to day 7 in all of the cultures.

It could not be unequivocally concluded from these results whether *G. metallireducens* could biodegrade PHAs or 3HB. When P(3HB) and Biopol films were supplied as the sole carbon source, a visible biofilm formed but no signs of degradation of the films (such as holes) were observed. It could be possible that some acetate, which served as growth substrate in all transfer and maintenance cultures, was carried over to the test cultures at time of inoculation, and used as the carbon source instead of the test substrate. Several serial transfers of acetate-grown cultures into medium with no carbon source were required before growth slowed down indicating that acetate was being transferred into the fresh medium.

4.2.3 Mixed Culture Biodegradation Studies Under Ferric Iron-Reducing Conditions

Iron-reducing bacteria such as *S. putrefaciens* have been detected and isolated from the spring water used as the inoculum for PHA biodegradation studies under sulfate-reducing conditions (F.D. Cook, personal communication). *S. putrefaciens* form distinctive orange, concave colonies on B10 agar plates (Semple and Westlake, 1987). However, very little iron-reducing activity was detected in spring water cultures amended with PHAs, 3HB, acetate and butyrate as less than 0.2% CO_2 and less than 4.0 µg/mL Fe(II) were measured in these cultures after 16 days incubation at room temperature. In contrast, cultures amended with yeast extract produced 2.7 ± 0.33% CO_2 and 243.8 ± 4.7 µg/mL Fe(II) after 16 days of incubation. The inoculum volume may have been too small and so uneven distribution of bacteria would have resulted. A rich source of carbon and other nutrients found in yeast extract allowed for the rapid growth of the bacteria found in the spring water.

A several day-old enrichment culture of spring water and spring water sediment in a ratio of approximately 1 to 1 under ferric iron-reducing conditions was used to inoculate new cultures. After 43 days of incubation the CO_2 production in PHAs-, 3HB-, acetate- and butyrate-amended cultures was less than 0.4%. Unamended cultures also produced this amount of CO_2. Cultures amended with yeast extract produced 0.67 ± 0.12% CO_2 after 43 days. Ferric iron reduction had occurred in these cultures as 683.4 ± 9.6 µg/mL

Fe(II) was measured after 43 days. Less than 60 μg/mL Fe(II) was detected in the other test cultures. Despite inoculation with an enriched spring water culture under ferric iron-reducing conditions, little or no growth or Fe(III) reduction occurred in cultures amended with PHAs, 3HB, acetate or butyrate. Yeast extract-amended cultures showed high iron-reduction but low CO_2 production.

Conflicting results were obtained when sewage sludge was used as the inoculum under iron-reducing conditions. After 56 days, high CO_2 production was measured in cultures amended with 3HB (6.5 ± 1.2% CO_2) and in cultures amended with yeast extract (8.6 ± 0.84% CO_2) (Figure 4.4) but Fe(II) production was less than 200 μg/mL for 3HB cultures and approximately 400 μg/mL for yeast extract-amended cultures (Figure 4.5). The converse was true for PHAs-, acetate- and butyrate-amended cultures. Levels of less than 3% CO_2 were detected in these cultures (Figure 4.4), yet greater than 750 μg/mL Fe(II) were produced after 56 days (Figure 4.5). Statistical analysis revealed that after 56 days the CO_2 production in yeast extract- and 3HB-amended cultures were significantly greater than in the unamended cultures. CO_2 production in P(3HB-co-15%3HV)-amended cultures was also significantly greater than the unamended cultures but significantly less than in yeast extract- and 3HB-amended cultures. The CO_2 production in acetate-, butyrate- and P(3HB)-amended cultures were not statistically different ($P<0.05$) from the unamended cultures. Fe(II) accumulation in acetate-, butyrate-, P(3HB)- and P(3HB-co-15%3HV)-amended cultures were not statistically different ($P<0.05$) from the Fe(II) accumulation in unamended cultures. However, yeast extract- and 3HB-amended cultures had accumulated Fe(II) in amounts signifcantly less than in the unamended cultures.

Table 4.1 summarizes the pH, CO_2 production, Fe(II) and VOA accumulation in the cultures after 81 days. It would be expected that high CO_2 levels under iron-reducing conditions would indicate an active population of iron-reducers and, therefore, high levels of reduced Fe(II) in the culture medium. The pH measurements showed that cultures with low CO_2 levels had high pH values (7.5 - 8.3) while those with high CO_2 levels had low pH values (6.0 - 7.2) (Table 4.1). CO_2 partitions into both the liquid or gaseous phases and the partition ratio depends on pH of the medium. At pH values of 6.0 -7.0, less than one-half of CO_2 is present in the gas phase, whereas at higher pH values of 7.0 - 9.0 less than 10% of the CO_2 is present in the gas phase (Fedorak *et al.*, 1982). Table 4.1 shows that the amounts of CO_2 that were dissolved in the culture fluid of 3HB- and P(3HB-co-15%3HV)-amended cultures were 3.0 ± 0.10 and 7.0 ± 0.40 μmol respectively. The pH

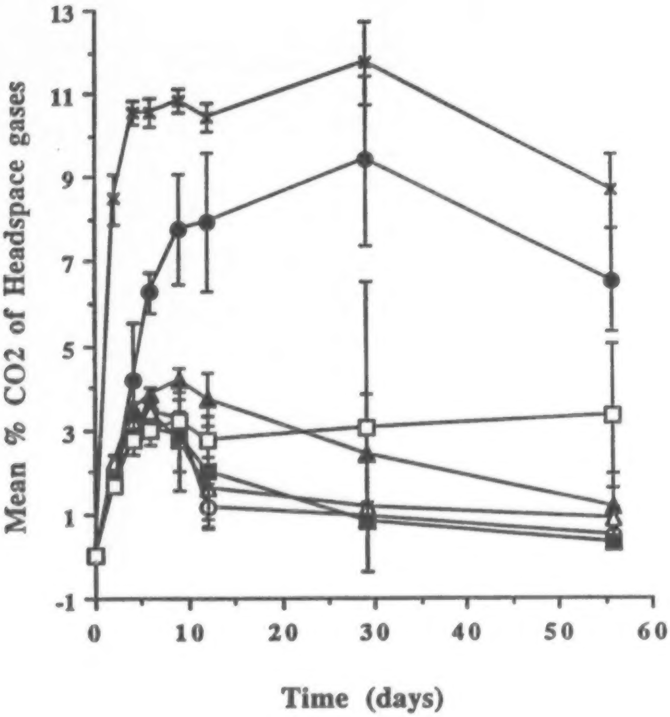


Figure 4.4. Mean cumulative CO_2 production from sewage sludge-containing cultures amended with the following substrates: -x- yeast extract; -o- unamended; -▲- acetate, -Δ- butyrate, -●- 3HB, -■- P(3HB) and -□- P(3HB-co-15%3HV). Error bars show one standard deviation.

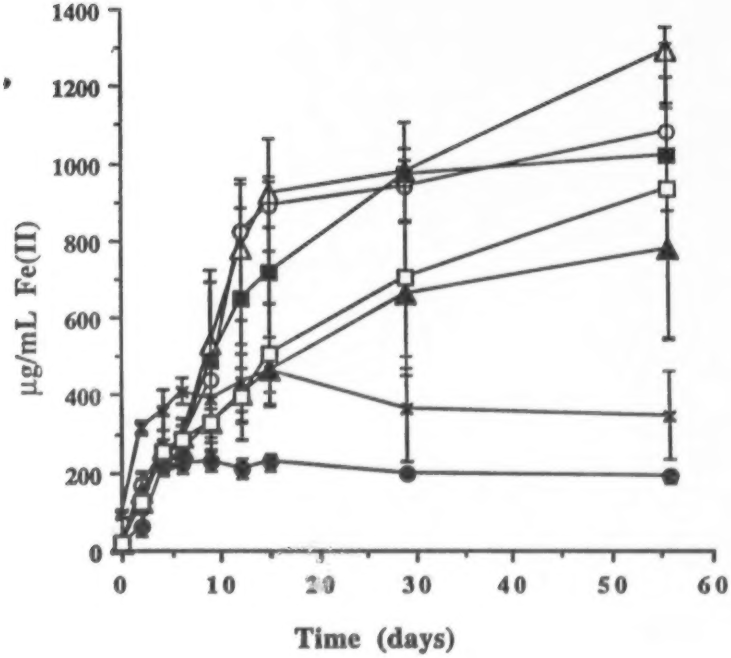


Figure 4.5. Mean cumulative Fe(II) production from sewage sludge-containing cultures amended with the following substrates: -x- yeast extract; -o- unamended; –▲– acetate, –Δ– butyrate, –●– 3HB, –■– P(3HB) and –□– P(3HB-co-15%3HV). Error bars show one standard deviation.

Table 4.1 Comparison of analytical results of sewage sludge-containing cultures incubated under iron-reducing conditions after 81 days.

Sample	pH	Fe(II)[a] mg/mL	% CO_2	μmol CO_2 in culture fluid	Total mM VOAs[b]
P(3HB)	8.14±0.07	0.90±0.11	0.88±0.23	12.0±0.06	10
Unamended	8.06±0.03	0.98±.0093	1.2±0.11	12.0±0.01	11
Butyrate	8.03±0.09	1.2±0.089	1.7±0.35	13.0±0.20	0
Acetate	7.93±0.16	0.86±0.066	1.9±0.86	13.0±0.06	66
Yeast Extract	7.16±0.13	0.33±0.11	11.6±1.5	13.0±0.10	58
P(3HB-co-15%3HV)	6.88±0.06	0.66±0.31	6.2±2.3	7.0±0.40	35
3HB	6.01±0.16	0.16±0.017	9.8±0.07	3.0±0.10	194

[a] Change in Fe(II) accumulation from time zero.
[b] VOAs include acetate, propionate and butyrate

values of these cultures fell in between 6.0 and 7.0. The amounts of CO_2 dissolved in the culture fluids of yeast extract-, acetate-, butyrate- and P(3HB)-amended cultures were very similar, either 12.0 or 13.0 μmol and statistical analysis revealed these amounts to be significantly greater ($P<0.05$) than the dissolved CO_2 found in the 3HB- and P(3HB-co-15%3HV)-amended cultures. The pH range of the yeast extract-, acetate-, butyrate- and P(3HB)-amended cultures was between 7.0 and 9.0.

Higher amounts of VOAs were present in the 3HB-, acetate- and yeast extract-amended cultures than in the other cultures. These cultures may, therefore, have bacteria present that were fermenting the substrates and producing acids which would result in the lowering of the pH. However, acetate-amended cultures had a pH value of 7.9 after 81 days of incubation. A stoichiometric equation for acetate consumption with $Fe(OH)_3$ reduction is given below (S. Goodwin, Univ. of Massachusetts, personal communication):

$$CH_3COO^- + 8Fe(OH)_3 + 16H^+ \rightarrow HCO_3^- + H_2CO_3 + 8Fe^{2+} + 20H_2O \qquad \text{Eqn. 4.1}$$

As 1 mol of acetate is consumed, 16 mol H^+ are consumed with 8 mol of Fe(III) being reduced. Therefore, a small amount of acetate consumption would result in a large pH increase. The actual reaction given in Eqn. 4.1 is likely more complex than written and forms of Fe(II) may be produced. Other stoichiometric equations for acetate consumption yielding $FeCO_3$ or Fe_3O_4 are given below (Roden and Lovley, 1993):

$$CH_3COO^- + 8Fe(OH)_3 + 6HCO_3^- + 7H^+ \rightarrow 8FeCO_3 + 20H_2O \qquad \text{Eqn. 4.2}$$

$$CH_3COO^- + 24Fe(OH)_3 \rightarrow 8Fe_3O_4 + 2HCO_3^- + 36H_2O + H^+ \qquad \text{Eqn. 4.3}$$

As equations 4.2 and 4.3 show, the final pH of the cultures depends on the form of Fe(II) produced.

4.2.4 Conclusions

The results from pure culture experiments with *S. putrefaciens* showed that this bacterium could not utilize PHAs or 3HB for growth under iron-reducing conditions. It was not possible to unequivocally demonstrate that *G. metallireducens* could utilize PHAs or 3HB under iron-reducing conditions.

It was possible to obtain an iron-reducing culture from spring water if yeast extract was added as the carbon and energy source. No active iron-reducing cultures were obtained from spring water when PHAs, 3HB, acetate or butyrate served as the sole carbon and energy source even when cultures were inoculated with an enriched culture of spring water. Therefore, the results from these experiments showed that iron-reducing bacteria were found in the spring water inoculum. However, the use of PHAs, 3HB, acetate and butyrate by bacteria coupled to iron reduction in the spring water could not be detected.

An active iron-reducing culture could not be obtained when anaerobic sewage sludge served as the inoculum. Cultures with high CO_2 production did not demonstrate a corresponding increase in Fe(II) due to Fe(III)-reduction. It was also not demonstrated whether PHA and 3HB metabolism could be coupled to iron-reduction.

The pH of the cultures changed over time even though the medium contained buffering components. It was shown that those cultures with a pH of 6.0 to 7.1 had a higher amount of VOAs in the culture fluid than in those cultures with a pH of 7.5 to 8.3.

4.3 Overall Summary and Conclusions

The results obtained from the experiments just described illustrate the difficulties in working with unknown consortia and trying to simulate environmental conditions in the laboratory. The large degree of variability in growth among the cultures even when the culture conditions and substrates were identical point to the fact that the low inocula volumes used led to uneven distribution of bacteria amongst the cultures. The bacterial numbers in the spring water may have been quite low initially. No enumeration of bacteria in spring water was done. The addition, however, of a rich source of nutrients such as yeast extract resulted in rapid bacterial growth, indicating bacteria were present in the sample of spring water. The other substrates may not have provided enough energy to support rapid growth.

Studies have shown that the complete oxidation of complex organic material coupled to Fe(III) reduction probably involves a consortium containing Fe(III)-reducing bacteria (Lovley and Phillips, 1988). Fermentative organisms in the consortium hydrolyze the complex organic matter to smaller compounds. These fermentative products are oxidized with the concomitant reduction of iron by a second group of bacteria. *S. putrefaciens*, a known iron-reducing bacteria, was not able to utilize PHA. This organism lacked depolymerases to break down the polymer, nor would it grow on the monomer 3HB. It was able to grow on lactate and pyruvate which are common fermentation end products. The carbon substrate range for *G. metallireducens* is quite broad and the organism has been shown to hydrolyze aromatic compounds (Lovley *et al.*, 1993). It is conceivable that PHAs could also be utilized by this organism, however, this could not be unequivocally proven from the experiments conducted.

SRB have been shown to use low molecular weight compounds as electron donors. Just as the iron-reducing bacteria, SRB rely on fermentative bacteria to break down complex organic matter into less complex, readily available products (Widdel, 1988). SRB are thus viewed as terminal degraders, much like methanogenic bacteria that form methane and CO_2 as final anaerobic products. However, a mixed fermentative bacteria and SRB consortium capable of degrading PHAs or 3HB could not be established with spring water or anaerobic sewage sludge as the inocula. The pure SRB culture experiments were set up under the assumption that the strains that were reported to synthesize P(3HB) could also degrade it exogenously. But it is, in the majority of cases, a foregone conclusion that bacteria which can synthesize P(3HB) cannot degrade exogenous P(3HB) since the extracellular depolymerase system of bacteria has been shown to be distinct from the intracellular depolymerase system (Dawes and Senior, 1973).

5. METHANOGENIC BIODEGRADATION OF PHAS

Galvin (1990) predicted that PHA biodegradation should occur at a faster rate in sewage sludge than in other environments such as sediments, activated sludge, soil and seawater. The high number of actively fermenting bacteria in sludge should be able to breakdown PHAs. Methanogenic bacteria cooperate with the chemoheterotrophic bacteria in degrading organic compounds. Since the biodegradation of organic matter is directly related to methane production in methanogenic environments (Bryant, 1979), it should be possible to correlate methane production to utilization of PHAs and other carbon sources by anaerobic cultures. Sediment from a local pond, rumen fluid and sewage sludge were used as inocula to test whether PHAs would be biodegradable in these environments.

5.1 Experimental Procedures

Sediment was inoculated (50% v/v) into 59-mL serum bottles using a wide-mouth pipette. Cultures were prepared in triplicate. The medium WR86 (Appendix 1.9) was used to give a final culture volume of 10 mL. PHAs were added at 500 mg/L to the serum bottles prior to addition of medium and sediment. PHAs tested included P(3HB), P(3HB-co-15%3HV), P(3HB-co-20%3HV) and P(3HB-co-26%3HV). The sodium salts of acetate, butyrate and 3HB were added at time of inoculation to a final amount of 400 mg/L. In addition, unamended cultures were set up to measure methane production from background carbon sources in the sediment. The cultures were incubated in the dark at either 35°C or 15°C after being adjusted to ambient atmospheric pressure with the manometer (see section 2.4.5). Methane production was initially measured as a percent of the headspace gases when various inocula were screened for their ability to degrade PHAs and other carbon substrates under methanogenic conditions. Once it was determined that the inocula contained PHA-degrading populations, new batch cultures were established and the total dry amount of methane produced in the cultures were determined. All methane results were analyzed statistically.

Rumen fluid from a dairy cow was collected from the Experimental Farm at the University of Alberta. The fluid was placed into the anaerobic hood and filtered first through 6 layers of cheesecloth and then twice through 12 layers of cheesecloth. This ensured that particulate matter, especially grass, was removed. The final filtrate was then removed from the hood and stored overnight on a lab bench in a container with the lid lightly screwed on to allow VOAs and methane to escape. This lowered the background

VOAs and methane levels in the rumen fluid before inoculation. Rumen fluid was inoculated into 59-mL serum bottles at 50% (v/v). Identical medium, substrates and substrate conditions as in the sediment cultures were used. The cultures were incubated in the dark at 35°C after being adjusted to atmospheric pressure with the manometer (see section 2.4.5). Methane production was measured and results analyzed statistically.

Domestic sewage sludge collected from the 26 ft depth of an anaerobic sludge digestor at the Gold Bar Wastewater Treatment Plant in Edmonton, Alberta was used as the source of methanogenic bacteria. The sludge was left standing overnight in a fumehood before inoculation. This was in order to allow excess VOAs and methane to escape. Cultures were prepared in triplicate. The culture volume was 10 mL, of which 5 mL was sewage sludge, the rest WR86 medium (see Appendix 1.9) and carbon source. The PHAs, P(3HB), P(3HB-co-15%3HV), P(3HB-co-20%3HV), were added at 500 mg/L. The sodium salts of acetate, butyrate and 3HB were added at time of inoculation to a final amount of 400 mg/L. In addition, unamended cultures were set up to measure methane production from background carbon sources in the sludge. The cultures were adjusted to ambient atmospheric pressure with the manometer (see section 2.4.5), and were incubated in the dark at 35°C. For the experiment comparing the biodegradation of various biopolymers, the biopolymers were added as dry powder, granules or strands all at 500 mg/L. Culture volumes were 10 mL in 59-mL serum bottles. Methane production was measured and results analyzed statistically.

5.2 Biodegradation of PHAs by Sediment Consortia

Four experiments were done using sediment collected from the pond at Hawrelak Park in Edmonton, Alberta. Two experiments used sediment collected in the fall before the pond froze. The sediment was stored at 4°C for several months before it was used. The first cultures set up were incubated for 30 days at 35°C, the same temperature as that for sludge cultures discussed later. Very inconsistent results, however, were obtained. In the second experiment, therefore, the cultures were incubated at 15°C which was more representative of the actual temperature of the pond sediment. There was a much slower microbial activity at this lower temperature and the experiment was carried out for 14 weeks. Figure 5.1 shows that no enhanced methane production above the control ($P<0.05$) was detected until 6 to 7 weeks of incubation. This occurred in the cultures amended with 3HB, P(3HB) and P(3HB-co-13%3HV). Further into the incubation, the methane yields in the P(3HB) and P(3HB-co-13%3HV) cultures increased very rapidly leveling off after 13 weeks at 0.11 mmol methane. Methane yields in 3HB-amended cultures only rose to a level of 0.03 mmol. Acetate-amended

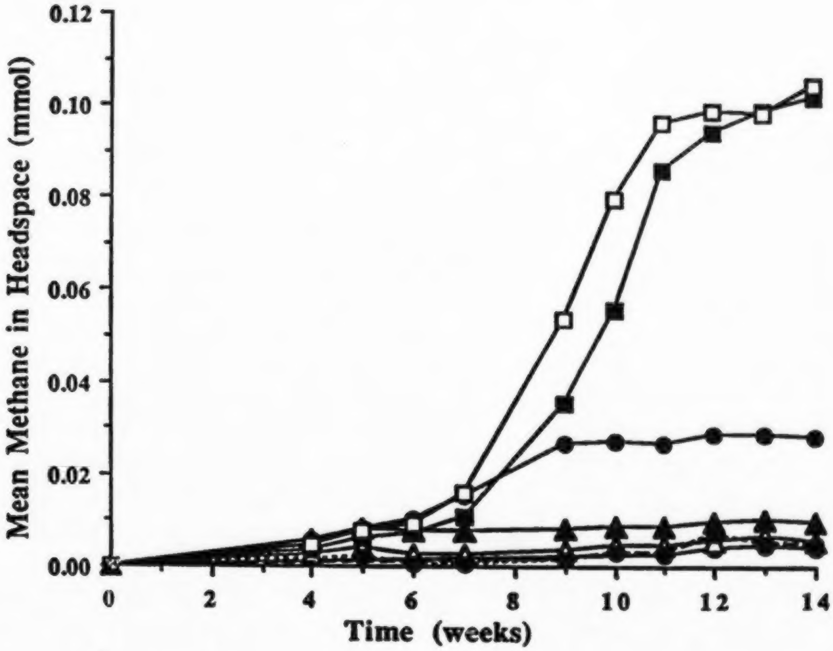


Figure 5.1. Mean cumulative methane production from sediment-containing cultures incubated at 15°C in the dark and amended with the following substrates: -▲- acetate, -Δ- butyrate, -●- 3HB, -■- P(3HB); -□- P(3HB-co-15%3HV); ---Δ--- P(3HB-co-20%3HV);.---●--- P(3HB-co-26%3HV) and -o- unamended.

cultures barely rose above the control in methane yield and methane was hardly detectable in cultures supplemented with butyrate, P(3HB-co-20%3HV) and P(3HB-co-26%3HV). It was not clear why no methane production from acetate- and butyrate- amended cultures occurred when methane production occurred from 3HB-, P(3HB)- and p(3HB-co-15%3HV)- amended cultures.

The variability in methane production among triplicate cultures was very low and, therefore, no error bars are shown in Figure 5.1 or in subsequent graphs presented in Chapter 5. The relative standard deviations of the cultures were below 10%. In contrast, the relative standard deviations of spring water-, sewage- and activated sludge-containing cultures under nitrate-, sulfate- and ferric iron-reducing conditions were greater than 10%.

The weight average molecular weights of the PHAs used in the experiments are shown in Table 5.1. These were used to determine the empirical formulae of the biopolymers which subsequently were used to calculate the carbon content of the PHAs. The proportions of carbon in the P(3HB) and P(3HB-co-20%3HV) preparations were determined by the Microanalytical Laboratory in the Department of Chemistry, University of Alberta. The measured carbon contents agreed closely with the calculated values. Based on the empirical formulae in Table 5.1 and the equation of Buswell and Mueller (1952);

$$C_nH_aO_b + [n - a/4 - b/2]H_2O \rightarrow$$
$$[n/2 - a/8 + b/4]CO_2 + [n/2 + a/8 - b/4]CH_4$$

the theoretical amounts of gas production from the degradation of 5 mg of the polymers and 4 mg of acetate, butyrate and 3HB could be calculated for the sediment- and sewage sludge-containing cultures. After 14 weeks of incubation the percent carbon from acetate, butyrate, 3HB, P(3HB) and P(3HB-co-13%3HV) converted to methane was 9.4, 1.1, 19, 43 and 43% respectively.

In the third and fourth experiments, cultures were inoculated with a fresh sample of sediment obtained during the summer and used within days of sampling. The sediment was either diluted and added to the flushed and stoppered serum bottles by means of a syringe or added directly to flushed but not stoppered serum bottles with a wide-mouth pipette. The culture sets with a direct addition of sediment showed higher microbial activity than the diluted sediment cultures, yet results in both experiments were similar. After 7 to 10 weeks of incubation at 15°C, enhanced methane production ($P<0.05$) was detected in cultures amended with P(3HB-co-13%3HV) and 3HB (Figure 5.2). At the end of the experiment, methane amounts reached between 0.14 to 0.16 mmol and 0.07 to 0.10 mmol in P(3HB-co-13%3HV)

Table 5.1 Characteristics of the PHAs used in the methanogenic experiments.

PHA	Molecular weight	Empirical formula	Percent Carbon	
			Calculated[a]	Found[b]
P(3HB)	1.0×10^6	$H\text{-}(C_4H_6O_2)_{12000}\text{-}OH$	55.8	55.5
P(3HB-co-13%3HV)	1.4×10^6	$H\text{-}(C_4H_6O_2)_{14000}\text{-}$ $(C_5H_8O_2)_{2100}\text{-}OH$	56.4	NA[c]
P(3HB-co-20%3HV)	1.5×10^6	$H\text{-}(C_4H_6O_2)_{14000}\text{-}$ $(C_5H_8O_2)_{3500}\text{-}OH$	56.8	56.3

[a] from formula

[b] from microanalysis

[c] NA, not analyzed

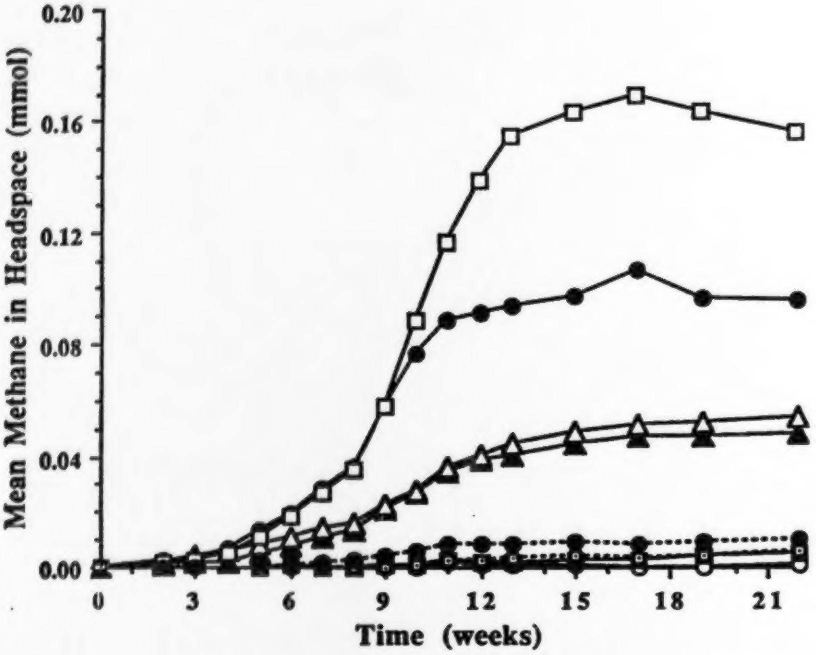


Figure 5.2. Mean cumulative methane production from cultures inoculated directly with sediment and incubated at 15°C in the dark. Cultures were amended with: -▲- acetate, -△- butyrate, -●- 3HB, -■- P(3HB); -□- P(3HB-co-15%3HV); ---▣--- P(3HB-co-22%3HV);---●--- P(3HB-co-26%3HV) and -o- unamended.

and 3HB cultures respectively. It was calculated that 57% of the carbon in P(3HB-co-13%3HV) and 76% of the carbon in 3HB was converted to methane after 15 weeks. Acetate-amended cultures (and butyrate-amended cultures to a lesser extent) also showed methane production, although levels only reached 0.03 to 0.04 mmol. These values were marginally significant when compared to the control cultures ($P<0.05$). After 15 weeks, 76% of the carbon in acetate was converted to methane and 34% of the carbon in butyrate was converted to methane. The rest of the cultures containing P(3HB) and other PHAs had no enhanced methane production over the control; methane amounts were lower than 0.01 mmol. A different sample of P(3HB) was used for these two experiments than for the initial experiment involving sediment. Something in this sample may have been inhibitory to the growth of the cultures as this would explain the observation that P(3HB) was readily utilized in the first experiment and not in the subsequent ones.

The microbial consortium in sediment was proven to be able to biodegrade P(3HB) and P(3HB-co-3HV) polymers of short side chain lengths as there were significant methane production over the non-amended control cultures ($P<0.05$). The monomeric form of P(3HB), 3HB, was readily utilized by the consortium as well. P(3HB-co-3HV) polymers of higher percent HV units than 15% were poorly, if not at all, degraded. This may be due to the fact that microbes with the proper depolymerase capable of utilizing these PHAs were not present in the consortium, or that the increased number of side chains blocked depolymerases from hydrolyzing the ester bond (Doi *et al.*, 1990).

5.3 Attempts to Demonstrate PHA Biodegradation by Rumen Fluid Consortia

5.3.1 Methane Production in Rumen Fluid Cultures

It became apparent from the initial experiments using rumen fluid as the inoculum, that there was no enhanced methane production ($P<0.05$) in the different cultures over the negative control cultures which received no added carbon substrate (Figure 5.3). This was not surprising considering the rumen microbial ecosystem. The major obstacle facing ruminal methanogens is being washed out from the rumen. Successive cell division of rumen microbes must occur within or before the turnover rate in the rumen pool in order to avoid washout (Boone, 1991). Two common pathways by which methane can be generated is by the reduction of carbon dioxide by H_2 and by the reduction of volatile fatty acids (VFAs) such

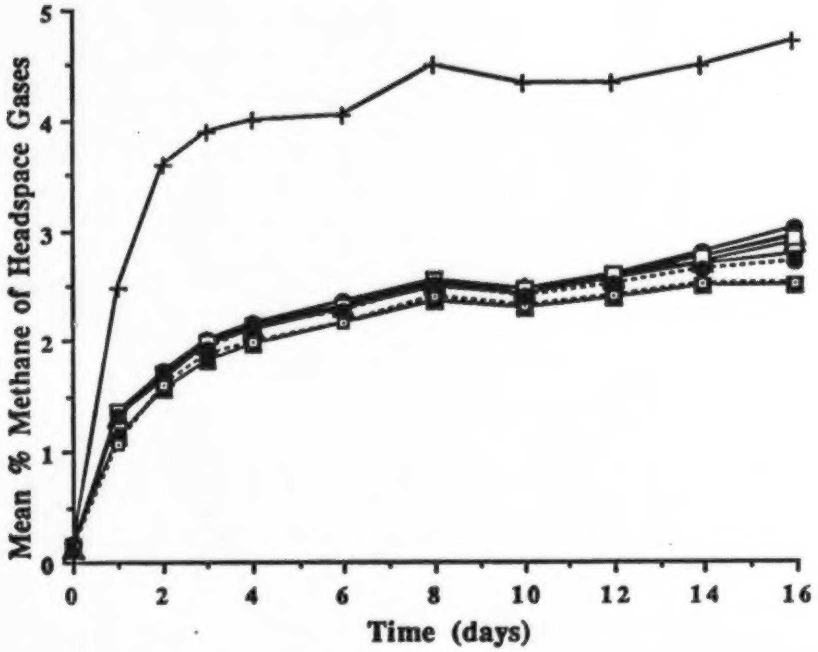


Figure 5.3. Mean cumulative methane production from rumen fluid-containing cultures incubated at 35°C in the dark. Cultures were amended with: -▲- acetate, -△- butyrate, -●- 3HB, -■- P(3HB); -□- P(3HB-co-15%3HV); ---⊡--- P(3HB-co-22%3HV); ---●--- P(3HB-co-26%3HV) and + H_2.

as acetate. The reactions, however, by which the rumen volatile acids can be further converted into methane do not provide sufficient energy to enable an aceticlastic methanogenic consortium to maintain itself against washout at rumen turnover rates. On the other hand, the energy from the reduction of H_2 enables hydrogenotrophic methanogens to grow at rumen fluid turnover rates. Therefore, the rumen methane comes from H_2-reduction of carbon dioxide and not from VFAs. VFAs are known to be absorbed by the ruminant through the intestinal epithelium (Garcia, 1990). The addition of 5 mL H_2 to one culture set demonstrated that hydrogenotrophic methanogens were present in the rumen fluid. Methane yields were significantly greater ($P<0.05$) in H_2-amended cultures than in non-H_2-amended cultures (Figure 5.3). The fact that no enhanced methane production occurred in acetate-amended cultures over the unamended cultures demonstrated that no aceticlastic methanogens were present in the rumen fluid.

5.3.2 Summary and Conclusions

Despite the fact that no conclusive evidence was obtained showing that PHAs were biodegraded by a rumen fluid consortium, irrefutable proof that some rumen fluid microbes are capable of degrading P(3HB) came from plating diluted rumen fluid onto P(3HB) overlay plates in the anaerobic hood. After a few weeks, zones of clearing appeared around colonies. This indicated that a P(3HB) depolymerase was being secreted by the bacteria. One of these organisms could also degrade PHA under aerobic conditions and was studied further as described in Chapter 6.

5.4 Biodegradation of PHAs by Sewage Sludge Consortia

5.4.1 Methane Production

Figure 5.4 illustrates the cumulative production of methane in the various sewage sludge cultures over a 21-day period. Fermentable substrates accompanied the anaerobic sludge inoculum and these yielded methane as observed in the unamended control cultures. The amounts of methane in the PHA-containing cultures and in the control cultures were the same after one day of incubation. By the third day of incubation, the amounts of methane in the test samples were significantly greater ($P<0.05$) than those found in the unamended cultures. Of the various polymers tested, cultures containing P(3HB-co-13%3HV) had the greatest methane production by day 3, while P(3HB)-containing cultures had the least amount of methane. On day 4, the methane production in the culture containing P(3HB-co-20%3HV)

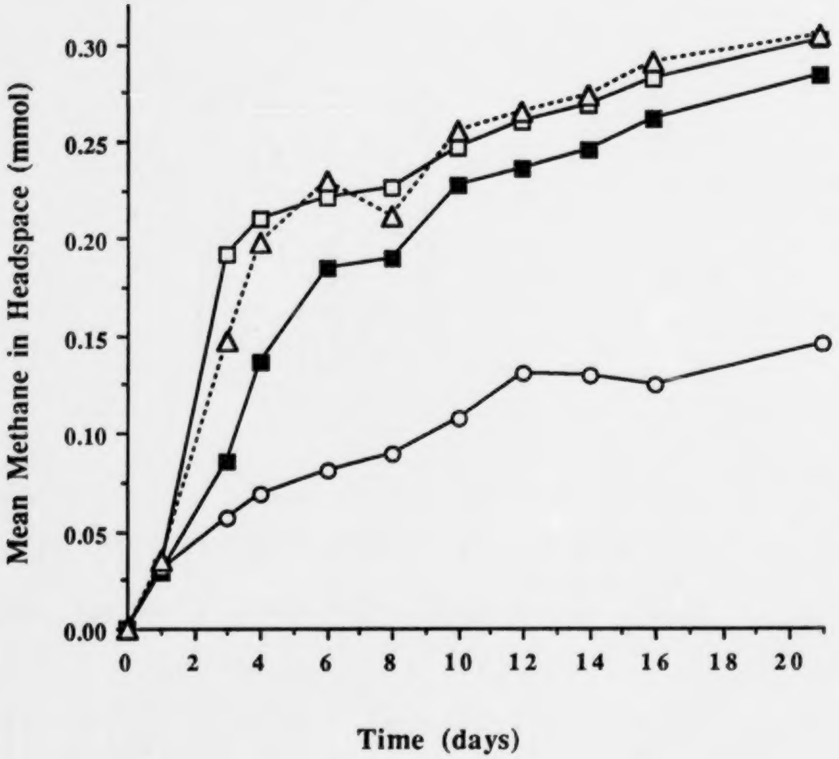


Figure 5.4. Mean cumulative methane production from sewage sludge-containing cultures under methanogenic conditions amended with: -■- P(3HB); -□- P(3HB-co-13%3HV);---Δ--- P(3HB-co-20%3HV) or -o- unamended.

did not differ significantly ($P<0.05$) from the P(3HB-co-13%3HV)-amended cultures. By the eighth day of incubation, no significant differences ($P<0.05$) in methane production among the test samples were detected. Clearly, the presence of PHAs in the test cultures yielded rapid methane production above that in the unamended control cultures. Figure 5.5 shows that conversion of 3HB and acetate to methane proceeded very rapidly. Indeed, the rate of methane production in cultures incubated with these substrates was significantly greater ($P<0.05$) than the rate of methane production in the unamended controls during the first day of incubation. Surprisingly, those cultures incubated with butyric acid showed no significant increase in methane production over the control cultures (Figure 5.5), even after 21 days of incubation.

Methane and total gas measurements taken from the sixteenth day of incubation revealed that, for all the polyesters, the measured values were near the theoretical values (Table 5.2). The total gas produced was 87%, 96% and 83% of the expected values for P(3HB), P(3HB-co-13%3HV) and P(3HB-co-20%3HV), respectively. The theoretical amount of methane produced from each substrate was within the range of the observed mean ± 1 standard deviation.

Strips of P(3HB) solvent-cast films were incubated in anaerobic sewage sludge at 35°C. After 10 days visible holes appeared (Plate 5.1). These holes were caused by microbial activity because no film deterioration occurred when the film was incubated in a sterile culture during the same time period. The films were prepared for scanning electron microscopy and the SEM pictures (Plate 5.2a) show that the film exposed to sewage sludge was colonized by bacteria. In some cases it appeared the bacteria had colonized depressions or pits found on the surface of the film. The SEM photograph of the sterile control film (Plate 5.2b) shows that the surface of the film was slightly uneven and pitted. The depolymerase activity of the bacterial colonies may have caused the pits as well, certainly Plate 5.1 shows that the bacteria were able to degrade right through the films.

5.4.2 Testing the Biodegradability of Various Polymers in Sewage Sludge Cultures Incubated Under Methanogenic Conditions

In addition to the above experiments, plastic materials from various sources were also subjected to methanogenic degradation by the sewage sludge consortium (Figure 5.6). The plastic materials tested included strips of a shampoo bottle made of PHAs (Biopol, ICI Ltd.), a plastic bag containing polyethylene and a magazine wrapper. The last two plastic materials

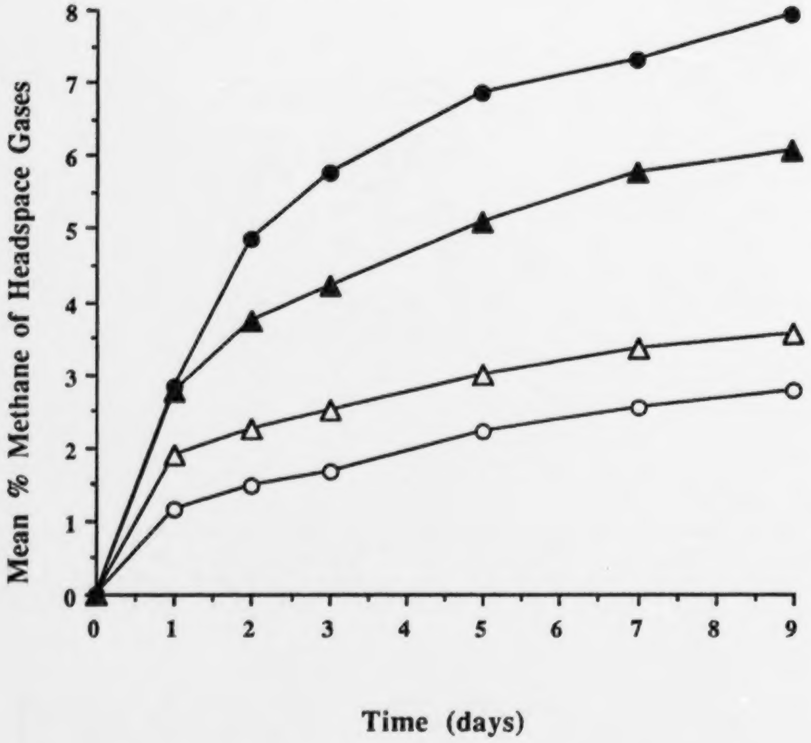


Figure 5.5. Mean cumulative methane production from sewage sludge-containing cultures under methanogenic conditions amended with:–▲– acetate, –Δ– butyrate, –●– 3HB or–o– unamended.

Table 5.2 Summary of P(3HB), P(3HB-co-13%3HV) and P(3HB-co-20%3HV) gas production plus carbon mass balance after 16 days of incubation with sewage sludge as inoculum.

	Total methane production (mmol)		Total gas production (mmol)		Conversion of substrate carbon to gas
Substrate	Measured	Theoretical	Measured	Theoretical	(%)
P(3HB)	0.14 ± 0.034	0.13	0.20 ± 0.12	0.23	87
P(3HB-co-13%3HV)	0.16 ± 0.033	0.14	0.23 ± 0.11	0.24	96
P(3HB-co-20%3HV)	0.17 ± 0.029	0.14	0.20 ± 0.11	0.24	83

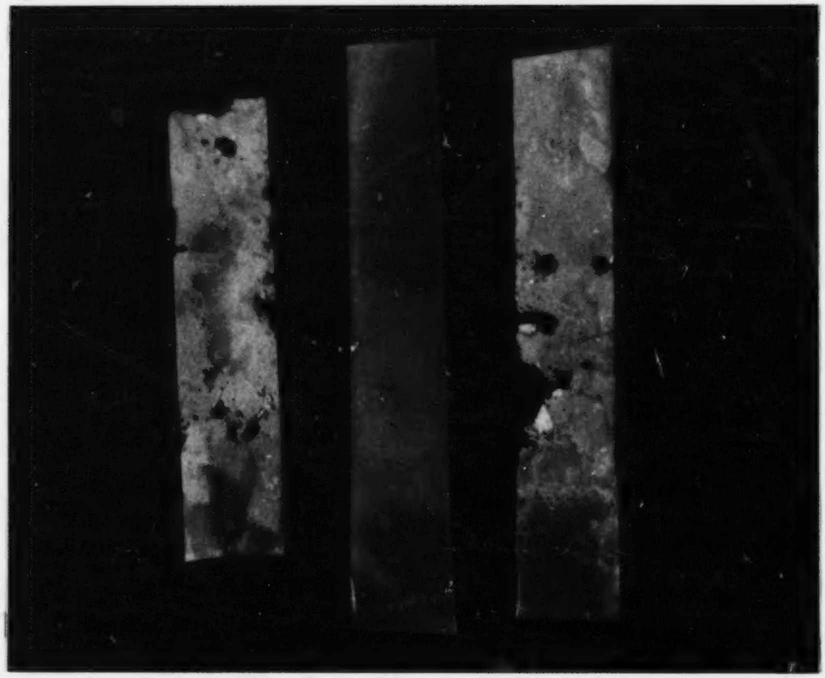


Plate 5.1. P(3HB) films after incubation in sewage sludge-containing cultures under methanogenic conditions for 10 days at 35°C. Sterile control, middle strip; test, outer strips. Original dimensions were 1 cm x 7 cm and 0.016 mm thick.

Plate 5.2. Scanning electron micrographs of P(3HB) films exposed to either: a) sewage sludge culture or to b) growth medium (control) for 10 days at 35°C.

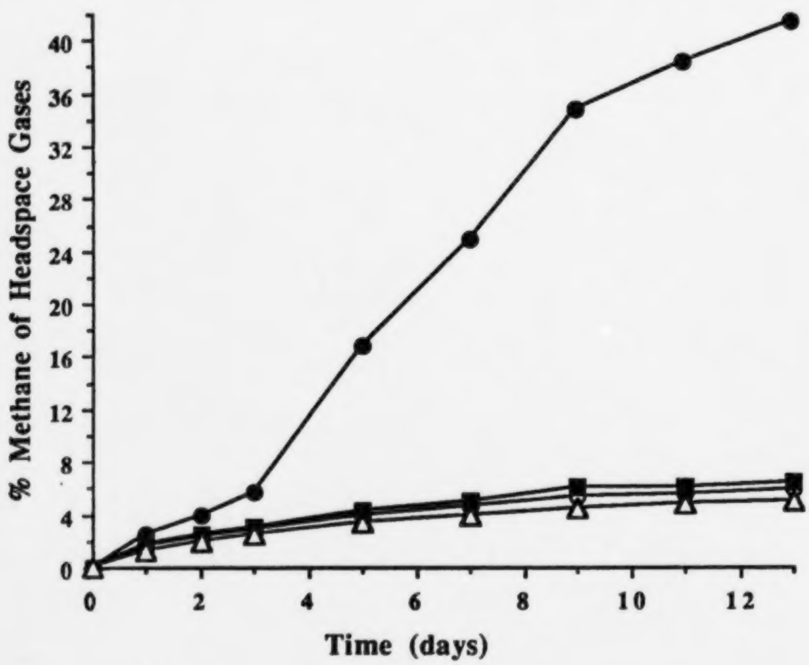


Figure 5.6. Mean cumulative methane production from sewage sludge-containing cultures amended with plastic strips of: -●- Biopol shampoo bottle; -■- plastic bag; -Δ- magazine wrapper and -o- unamended.

were tested since a statement on the plastic bag claimed it contained patented agents which enhances film deterioration in landfill sites and a statement on the magazine wrapper claimed it was biodegradable. The exact composition of the plastic bag and magazine wrapper is not known yet their biodegradability under methanogenic conditions was compared to that of the Biopol sample. The Biopol shampoo bottle was converted into methane very rapidly. In contrast, the methane yields from the cultures with the plastic bag and wrapper strips were indistinguishable ($P<0.05$) from the unamended cultures. These results show that a commercial sample of PHAs was readily degraded in sewage sludge under methanogenic conditions, yet other plastics claiming to be biodegradable were not.

A comparison of the degradation of various biopolymers, including alginate, starch, DNA, cellulose, chitosan, and collagen, to that of P(3HB) under methanogenic conditions was performed. Figure 5.7 shows that most of the soluble biopolymers such as starch and DNA were rapidly degraded, whereas the insoluble biopolymers, P(3HB), cellulose and chitosan, showed a lag period of 3 to 4 days before significant amounts of methane were detected.

5.4.3 Summary and Conclusions

Although it has been reported that PHAs degrade in sewage sludge, no sound data exists in the literature to back the reports up. The data presented in this thesis were the first unequivocal demonstration that P(3HB) and copolymers of P(3HB-co-3HV) were biodegradable under methanogenic conditions by sewage sludge consortia. The substrates were rapidly fermented to methane and CO_2 during the 21-day test period. The methane yields were close to the expected yields, and greater than 80% of the substrate carbon could be converted to total gas. SEM analysis (Plate 5.2a) revealed colonization by anaerobic bacteria on the surface of P(3HB) films. In some instances bacteria had colonized pits or had created these pits through the action of their depolymerases. Large holes in the films must be due to bacterial action since no such holes were found in sterile controls (Plate 5.1). Also, PHA was shown to be truly biodegradable unlike plastics claiming to be biodegradable (Figure 5.6). These plastics were not biodegraded in sewage sludge despite the high number of actively degrading microorganisms.

In the experiment whose results are shown in Figure 5.4 and 5.5, the enhanced methane production from 3HB was evident after 1 day of incubation, indicating that this monomer was readily fermented to methane. In contrast, after 1 day of incubation, the

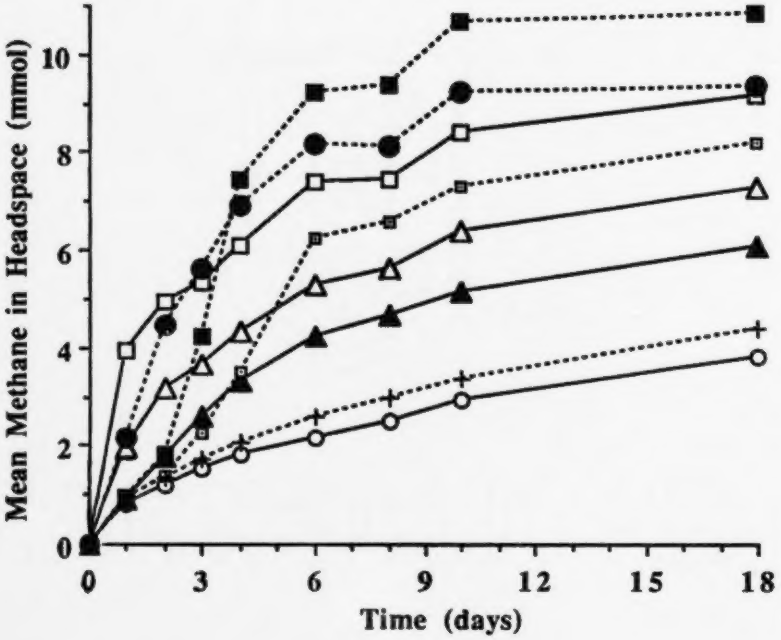


Figure 5.7. Mean cumulative methane production from sewage sludge-containing cultures amended with various biopolymers: -△- aliginate, -□- starch; -▲- DNA; ---◘--- cellulose, ---+--- chitosan; ---●---collagen, ---■---P(3HB) and -o- unamended.

amount of methane in the PHA-containing cultures was not greater than that in the unamended control culture. This indicates that the readily fermentable monomer was not present and suggests that the hydrolysis of the P(3HB) and P(3HB-co-3HV) polymers was the rate-limiting step in methanogenic degradation of the PHAs. As Figure 5.7 shows, the hydrolysis of insoluble polymers was slower than the hydrolysis of soluble polymers. Cellulose hydrolysis has been shown to be the rate-limiting step in anaerobic digestion by other researchers (Eastman and Ferguson, 1981; Gujer and Zehnder, 1983; Pfeffer, 1974).

It had been expected that butyric acid would have been readily utilized and converted to methane by the consortium. But Wolin (1982) states that $\Delta G°' = +48.1$ kJ for the oxidation of butyric acid to acetic acid and H_2 and $\Delta G°' = -35.1$ kJ for the oxidation of 3HB to acetic acid and H_2. Thus, 3HB would be more amenable to oxidation than butyric acid. A 3HB-fermenting bacterium, *Ilyobacter polytropus*, has been observed in low numbers in anaerobic digestor sludge (Stieb and Schink, 1984). This bacterium ferments 2 mol of 3HB to 2 mol of acetate and 2 mol of butyrate. It is unlikely that this type of fermentation was significant in the cultures set up in this study. If butyrate was produced, it would probably accumulate in the medium because of the inability of the sludge inoculum to produce methane from this acid (Figure 5.5). Thus, at most only 50% of the carbon from P(3HB) would be converted to gas. However, the results in Table 5.2 show that 87% of the carbon from P(3HB) was recovered as gas.

P(3HB) accumulation has been found in cells of the anaerobic syntrophic bacterium, *Syntrophomonas wolfei* (Amos and McInerney, 1991). This bacterium can also degrade C_4 to C_8 straight-chain fatty acids to acetate and H_2 or to acetate, propionate and H_2 (Wofford *et al.*, 1986). These reactions, however, are energetically unfavorable unless the H_2 concentration is maintained at a very low level by H_2-using methanogens (McInerney *et al.*, 1979).

The mechanism of P(3HB) depolymerization under aerobic conditions is known to occur in two steps (Shirakura *et al.*, 1986). P(3HB) is broken down to dimer and eventually to monomer forms and the monomers are then absorbed by the microorganisms (Shirakura *et al.*, 1986; Tanio *et al.*, 1982). Overall, the biodegradation of P(3HB) and P(3HB-co-3HV) under methanogenic conditions in sewage sludge would most likely involve the initial hydrolysis of PHA to its monomeric units, and the conversion of the monomers (such as 3HB) to acetate and H_2 by the H_2-producing acetogenic bacteria (such as *S. wolfei*) in syntrophic association with H_2-using methanogens (Mackie *et al.*, 1991). Ultimately, the methanogens then complete the degradation of PHAs with the conversion of acetate to methane and CO_2.

5.5 Overall Summary and Conclusions

PHAs were shown to be biodegradable under methanogenic conditions in sewage sludge and sediment. As predicted by Galvin (1990), PHAs were rapidly biodegraded in sludge and at a faster rate than in sediment. A greater percent of the carbon in PHAs was converted to methane when exposed to sewage sludge than to sediment. The lower temperature and most likely lower diversity and numbers of bacteria in sediment probably accounted for the slower biodegradation of PHAs with this inoculum. The disposal of PHAs into a sewage sludge system represents a completely closed system where the waste polymers made by bacteria are fed back to bacteria (Logsdon, 1989). Methane gas may be recovered as fuel during the process.

The biodegradation of PHAs in rumen fluid could not be unequivocally demonstrated. The high organic background in rumen fluid interfered with analytical measurements of methane and of VFA accumulation. The fact that a PHA-degrading microorganism was isolated from rumen fluid suggested that PHAs could be indeed biodegradable in this environment. The breakdown product of PHAs would most likely be absorbed by the animal. This raises the potential of PHAs serving as a possible food source for ruminants.

6. ISOLATION AND CHARACTERIZATION OF A PHA-DEGRADING ORGANISM

The purpose of this section was to isolate PHA-degrading microorganisms from the various anaerobic environments tested and to study their depolymerizing activities. To facilitate experiments, facultative anaerobic bacteria were isolated so that enzyme reactions and assays could be performed aerobically. Numerous methodology problems with enzyme assays were encountered. Part of this section, therefore, reviews the methods tried and possible reasons why they failed.

6.1 Experimental Procedures

In order to isolate PHA-degrading organisms from rumen fluid and sewage sludge, dilution blanks of 9 mL of 0.3 mM phosphate buffer (pH 7.0) with 0.1% sodium thioglycolate and 0.1% methylene blue in Hungate tubes were prepared. These tubes were placed into the anaerobic hood and allowed to reduce as indicated by the disappearance of a blue color from the solutions. A dilution series of 10^{-2} to 10^{-7} was prepared with rumen fluid and sewage sludge and 0.1 mL from each dilution was spread onto BHI-PHA overlay plates (see Appendix 1.10) so that a dilution range of 10^{-3} to 10^{-8} was achieved. The plates were incubated at 30°C and checked periodically for isolated colonies that could clear PHA from the overlay. Zones of clearing were usually observed within 1 week. Plates with such colonies were removed from the anaerobic hood and the colonies tested to see if they could grow aerobically at 28°C on PHA overlay plates (see Appendix 1.11).

Four different assay systems for measuring depolymerase activity that were tested are described here. In one method (Jendrossek *et al.*, 1993) PHA suspensions were prepared by mixing together 12.5 mL distilled water and 0.3% (w/v) PHA granules. The suspension was shaken overnight on a rotary shaker (Gyrotory Water Bath Shaker, Model G76, New Brunswick Scientific at 175 rpm and room temperature) to disperse the granules. The suspension was sonicated for 10 min (Braun Sonic 2000, B. Braun, intermediate probe at low setting, 0.035 Watts) to make sure any clumps of PHA were broken up, 12.5 mL glycerol was then added and the suspension sonicated for another 10 min. This gave a suspension of approximately 180 μg PHA/mL. The stability of the resulting suspension was measured at 650 nm for 60 min at 35°C.

The depolymerase assay system given below is the one used for the attempt to measure depolymerase activity from concentrated culture supernatant. Supernatant (18 mL) from 3-day-old *Staphylococcus* sp. culture grown on 0.2% (w/v) P(3HB-co-15%3HV) was concentrated 9-fold with Centricon-30 concentrators (Amicon, Inc.). The assay system was comprised of the following components:

Components	Control[a] (µL)	Test (µL)
Glycerol/Distilled water (1:1)	840	840
100 mM Tris HCl[b] pH 8.0	100	100
P(3HB-co-3HV) suspension	60	60
Distilled water	100	
Concentrated Supernatant		100

[a] The control measured the stability of the P(3HB-co-3HV) suspension.

[b] From 1M stock solution.

The components were added in order of appearance in the table and mixed to ensure the P(3HB-co-3HV) granules were suspended. Turbidity at 650 nm was measured immediately and read every 2 min for 60 min.

The second method was a modification of the first method of Jendrossek *et al.* (1993) described above. The PHA suspension was prepared as before. Clean, scratch-free 13mm x 100 mm screw-capped tubes were used. The assay solution consisted of the following components:

Components	Blank (mL)	Control[d] (mL)	Test (mL)
100 mM Tris HCl[a]	0.3	0.3	0.3
PHA Suspension		0.1	0.1
Supernatant[b]			0.2
Mineral medium[c]	2.7	2.6	2.4

[a] From 1 M stock solutions at pH 7.5, 8.0, 8.5 or 9.0.

[b] Supernatant was from a 2-week-old culture grown on P(3HB) and P(3HB-co-3HV).

[c] Semidefined *Staphylococcus* medium (Appendix 1. 12).

[d] Control measured stability of the PHA suspension.

The components were added to the tubes, mixed together and the turbidity read at 650 nm, every 30 min for 5 h.

In the third method (Gilmore *et al.*, 1990), melted agarose was added to the assay system to a final concentration of 0.25% (w/v). It was intended that once the agarose solidified it would prevent the PHA granules in the suspension from settling. A 100 µg/mL PHA suspension was made by adding 50 mg PHA to 25 mL distilled and deionized water and shaken overnight as described in the previous section. The suspension was sonicated for a total of 10 min.

The assay solution consisted of the following components:

Components	Blank	Control[c]	Test 1	Test 2
10 mM Tris HCl[a]	20 µL	20 µL	20 µL	20 µL
Distilled water	2.08 mL	1.98 mL	0.98 mL	1.48 mL
Supernatant[b]			1 mL	0.5 mL
PHA Suspension		0.1 mL	0.1 mL	0.1 mL
Agarose	0.75 mL	0.75 mL	0.75 mL	0.75 mL

[a] From 1 M stock at either pH 7.5 or 8.0.
[b] Taken from 1 week old culture grown on PHAs
[c] Control measured stability of the PHA suspension.

The components were added to clean, scratch-free 13 mm x 100 mm test tubes. Agarose was the last component to be added to the assay tube upon which the tubes were then mixed a final time and the agarose allowed to solidify for 90 s. The turbidity at 660 nm was measured immediately after 10 min intervals during incubation at 30°C.

In the fourth method (Janssen and Schink, 1993) the assay was divided into two parts in order to measure depolymerase activity found in the culture supernatant and depolymerase activity found associated with the PHA granules. To set up the assay measuring depolymerase activity in the supernatant, 20 mg PHA as dry powder and 5 mL of uninoculated culture medium were added to a screw-capped tube and autoclaved to sterilize. At sampling time, 5 mL of culture supernatant was filter sterilized through a 0.45

µm filter into the assay tube containing the sterile PHA granules and medium. To prevent any bacterial growth, a solution of sodium azide was added to a final concentration of 10 mM. Granule-associated depolymerase activity was measured by aseptically adding the remaining supernatant and granules of the culture used for the supernatant-associated depolymerase assay to a sterile screw-capped tube. Approximately full recovery of the 20 mg of granules in each culture was expected. The granules were washed twice in sterile, unioculated medium. The supernatant was discarded and 10 mL of sterile, uninoculated medium was added. To prevent any bacterial growth, a solution of sodium azide was added to a final concentration of 10 mM. The assay tubes were incubated at 28°C. At sampling times, 1 mL of the assay mixture was removed and methanolysis performed as described in section 2.4.7 in order to detect 3HB production

Staphylococcus sp. was grown in semidefined *Staphylococcus* medium (see Appendix 1.12) with each of the following substrates as sole carbon source: 56 mM glucose, 0.1% (w/v) P(3HB), and 0.1% (w/v) P(3HB-co-15%3HV). Glucose was added separately from a sterile stock solution after the medium had been sterilized. The PHAs were added as dry powders to the flasks and were sterilized with the medium. Culture volumes were 200 mL in 500-mL flasks. Cultures were inoculated with a 5% (v/v) inoculum that had been pregrown for 48 h in glucose-amended semidefined *Staphylococcus* medium, and were incubated at 28 to 30°C for 48 h with agitation at 175 rpm on a New Brunswick Scientific Co. model G-10 platform shaker. At each sampling time, 5 mL of culture fluid was removed for total cellular protein determination using the method of Lowry *et al.* (1951). As well, 1 mL of culture supernatant was analyzed for 3HB as described in section 2.4.7.

To measure depolymerase activity the method of Janssen and Schink (1993) was used (see previous section). Several cultures of 20 mL in 50-mL flasks were prepared, with 0.1% (w/v) P(3HB), and 0.1% (w/v) P(3HB-co-15%3HV) as the sole carbon sources. Cultures were grown under the same conditions as for the growth study described above and a culture was removed at each sampling point.

6.2 Isolation and Characterization of PHA-Degrading Organisms

Isolated colonies able to produce clear halos on P(3HB) and P(3HB-co-3HV) overlay plates under anaerobic conditions were tested to determine whether they could grow and degrade these polymers aerobically. Several strains were still viable and able to degrade

PHAs after being removed from the anaerobic hood. Using the API strips, the following bacteria were identified: *Klebsiella oxytoca*, isolated from sewage sludge; a possible *Pseudomonas* sp. or *Alcaligenes* sp. from sewage sludge; and *Escherichia coli*, isolated from rumen fluid. One isolate from rumen fluid was unable to be identified using the API strips and was sent to the Alberta Environmental Centre in Vegreville, Alberta for identification. The strain was identified as a *Staphylococcus* sp. This *Staphylococcus* species was chosen for further studies as it had the most consistent and most active depolymerase and was able to grow on P(3HB) as well as P(3HB-co-3HV). Plate 6.1 shows the zone of clearing around a *Staphylococcus* sp. colony after 3 days growth on a P(3HB-co-15%3HV)-overlay plate.

6.3 Testing of Various Depolymerase Assays

The most commonly cited assay to measure depolymerase activity is a turbidimetric method using PHA granules and was described by Tanio *et al.* (1982) for example. The method relies on obtaining a stable suspension of granules and then measuring the loss of turbidity of this suspension due to the degradation of polymer and solubilization of the monomer products. Slight modifications to this method have been reported. The method of Jendrossek *et al.* (1993) was tried in this study, however, the granules would not remain in suspension for very long as, within minutes, decreases in turbidity in suspensions of PHB granules were noted. The addition of crude supernatant from *Staphylococcus* sp. cultures grown on P(3HB) had no affect on the assay. Even after the supernatant was concentrated 9-fold no decrease in turbidity in the assay systems within 60 min were detected. The assay period may have been too short for significant changes in absorbance to occur.

The turbidimetric assay of Jendrossek (1993) was modified so that the assay could be carried out for longer periods of time than just 1 h. The assays were set up in 13 mm x 100 mm clean screw-capped tubes that could fit into the cuvette holder of a spectrophotometer (NovaSpec, LKB Biochem). At each absorbance measurement, the assay tubes were mixed with a vortex to resuspend the granules. This caused too much turbulence, however, and the absorbance readings were very scattered and inconsistent. As well, the volume of crude enzyme added may have been too small for the detection of activity.

Attempts to stabilize the suspension were met with mixed results. The addition of glycerol in various amounts had no affect on stability. Adding melted agarose to a final amount of 0.25% (Gilmore *et al.*, 1990) prevented the P(3HB) granules from settling down within 60 min (a slight decrease in turbidity occurred after 60 min). Supernatant from

Plate 6.1. *Staphylococcus* sp. colony producing a zone of clearing on P(3HB) overlay plates. Halo appeared within 3 days of incubation.

Staphylococcus sp. cultures taken from various times during growth on P(3HB) added at different volumes to the assay system never caused a significant decrease in absorbance over the control assay tube which contained only P(3HB). In fact, the absorbance often increased with time when supernatant was added. It is not known why this occurred. The effect of pH was investigated but since no depolymerase activity could be measured at any pH tried, no reliable conclusions could be made about pH effect.

Janssen and Schink (1993) reported a new method of measuring P(3HB) depolymerase activity in cultures of *Ilyobacter delafieldii*. They were able to determine the location and amount of enzyme activity in their cultures by measuring the accumulation of 3HB over time in supernatant fractions and P(3HB) granule fractions in essentially cell-free assay systems. Duplication of this method with some modifications resulted in observing an increase in 3HB release from PHA over time. A major source of error in this method was the presence of PHA granules in the sample before methanolysis. Great care had to be taken, such as centrifuging samples to pellet particulate matter, to ensure that no granules were methanolyzed as this would cause an increase in 3HB amount and lead to a false positive value.

6.4 *Staphylococcus* sp. Growth Studies and Depolymerase Activities

Figures 6.1 and 6.2a and 6.2b show the growth curves of *Staphylococcus* sp. when glucose, P(3HB) or P(3HB-co-15%3HV) were added as sole carbon sources. No growth lag was observed and some growth likely occurred from the casamino acids present in the medium. Growth occurred within 36 h on all substrates at which time the stationary phase was reached. Although the amount of substrate that remained in the cultures was not determined, PHA granules were still visible in the cultures after 36 h. A greater amount of cellular protein was measured when glucose served as substrate than when the PHAs did. This suggests perhaps that some component in the medium became limiting in the PHA cultures before the PHAs were totally metabolized. Figures 6.2a and 6.2b also show the accumulation and disappearance of 3HB in the PHA cultures over a 54 h time span. Since no 3HB was detected in the medium of glucose-grown cultures, the release of 3HB in the PHA cultures must have been from the biodegradation of PHAs. The disappearance of 3HB from the culture medium was due to the uptake of 3HB into the cells. There was a lag of about 8 to 10 h before 3HB was detected. Presumably this lag was due to the time needed for the secretion of the depolymerase by the *Staphylococcus* sp. cultures. 3HB levels began to drop

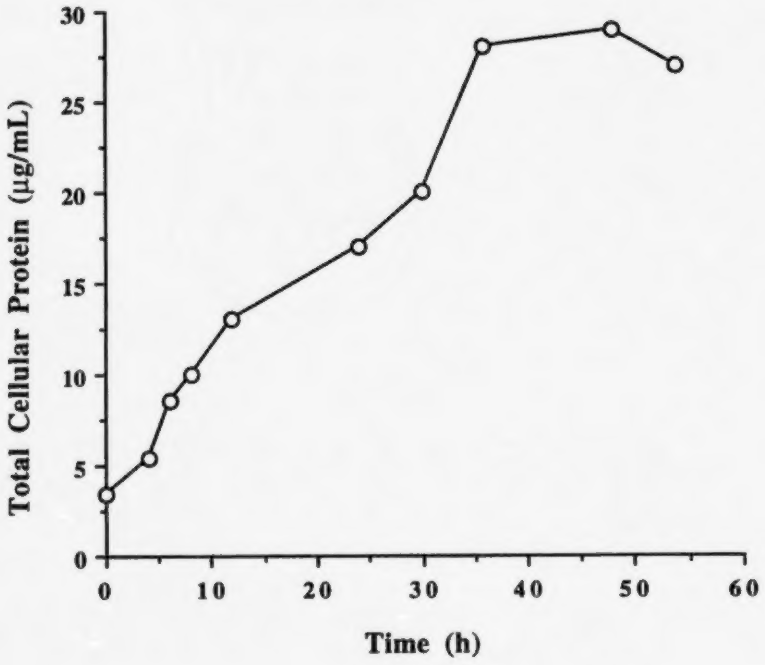


Figure 6.1. Growth curve of *Staphylococcus* sp. with glucose as carbon substrate.

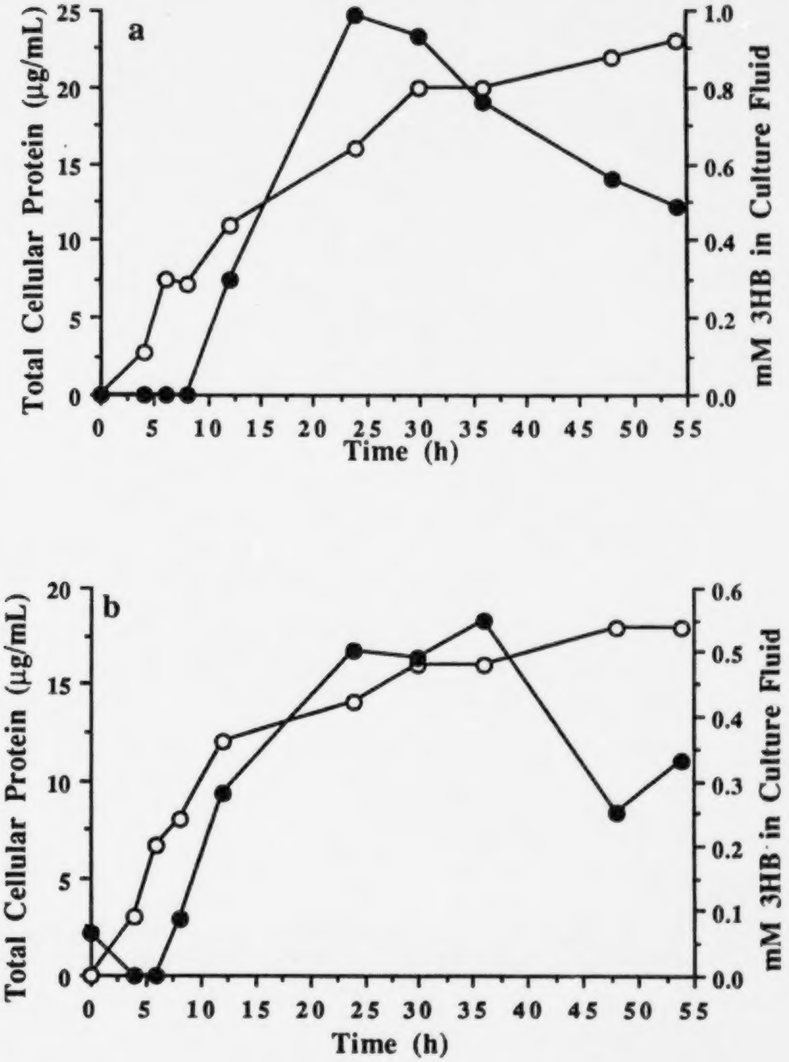


Figure 6.2. Growth curves and 3HB accumulation of *Staphylococcus* sp. with a) P(3HB) and b) P(3HB-co-15%3HV) as carbon substrates. symbols: -o- cellular protein, -●- 3HB.

after 30 to 35 h of growth, roughly corresponding to the beginning of the stationary phase of the cultures. These results were very reproducible.

New cultures were grown on P(3HB) and P(3HB-co-15%3HV) and at four times during the growth curve, the cultures were analyzed for depolymerase activity in both the culture supernatant or associated with the PHA granules. The times chosen for sampling were 12 h which represented the exponential growth phase, 24 h and 30 h which represented approximately the late exponential and early stationary phases respectively and 48 h which represented the stationary phase. The purpose of sampling at these different times was to determine when the maximum depolymerase activity occurred. Figures 6.3a and 6.3b show the 3HB release in the P(3HB) and P(3HB-co-15%3HV) granule assay systems. In the assay system, most of the 3HB was liberated from the polymer granules during the first 6 to 12 h, after that the production of 3HB stopped. A maximum of 0.4 mM 3HB was released when 0.3 g P(3HB) or 0.4 g P(3HB-co-15%3HV) was present in the assay systems. No 3HB was detected at the start of the assay period except in the 24- and 30-h samples given P(3HB-co-15%3HV) granules (Figure 6.3b). Approximately 0.35 mM 3HB was measured in these samples and most likely arose from experimental errors such as the inadvertant methanolysis of PHA granules present in the reaction tube. The rate of 3HB release was found to be similar from each time point sampled during the growth of the culture. Therefore, the depolymerase seems to be active during the exponential phase through to the stationary phase.

Figure 6.4 shows the results obtained when depolymerase activity associated with washed P(3HB-co-15%3HV) granules was monitored over a 12-h period. The granules came from a 24-h-old culture of *Staphylococcus* sp. growing on P(3HB-co-15%3HV). No 3HB was detected during the first 3 h, however, a good correlation between 3HB release and time was achieved between 4 and 12 h with a correlation value (r) of 0.97. The rate of 3HB release was determined to be 0.024 mM 3HB/h.

Most of the enzyme activity was granule-associated because no enzyme activity was detected in the supernatant of P(3HB)-grown and P(3HB-co-15%3HV)-grown cultures even if the assay time was extended to 48 h. This was consistent with observations of Mukai *et al.* (1993a) who found from depolymerase mechanism and kinetic studies that depolymerases adsorb onto the surface of the polymer. Although no 3HB released from P(3HB) granules was detected during the first 10 h, 3HB release from P(3HB-co-15%3HV) granules was observed between 4 to 12 h of assay incubation. This difference in rates may be a result of

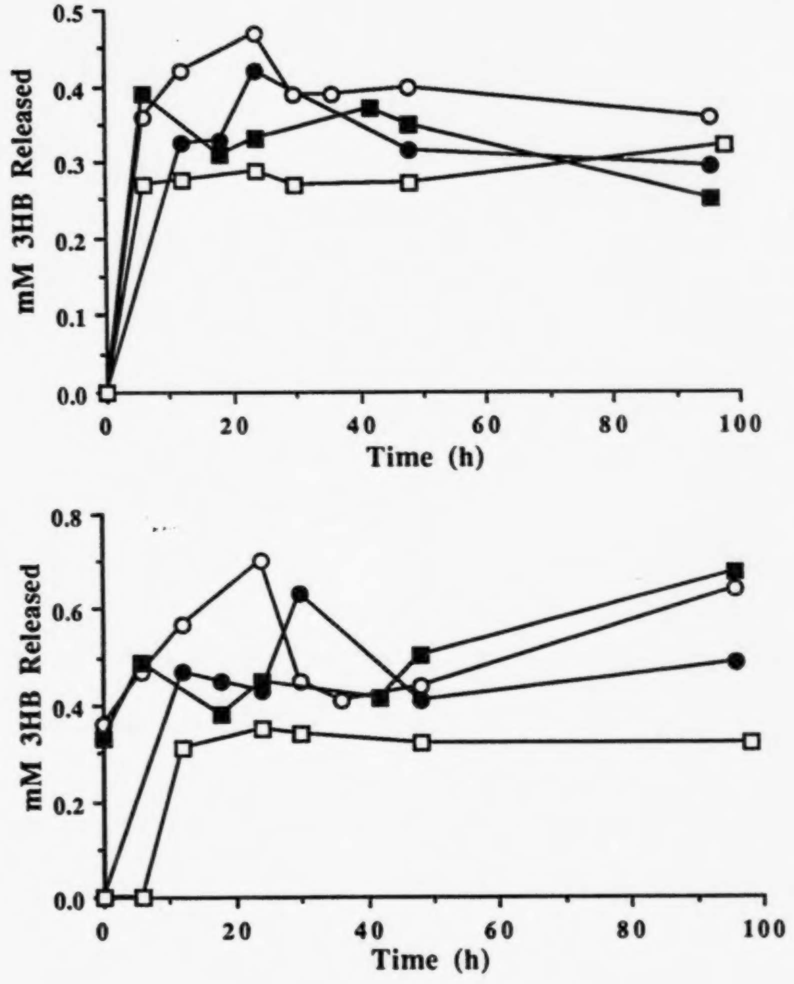


Figure 6.3. 3HB released in a) P(3HB) and b) P(3HB-co-15%3HV) granule assay systems. Sampling times for assay were: -●- 12h; -o- 24h; -■- 30h and -□- 48h in incubation of *Staphylococcus* sp. cultures grown on P(3HB) and P(3HB-co-15%3HV).

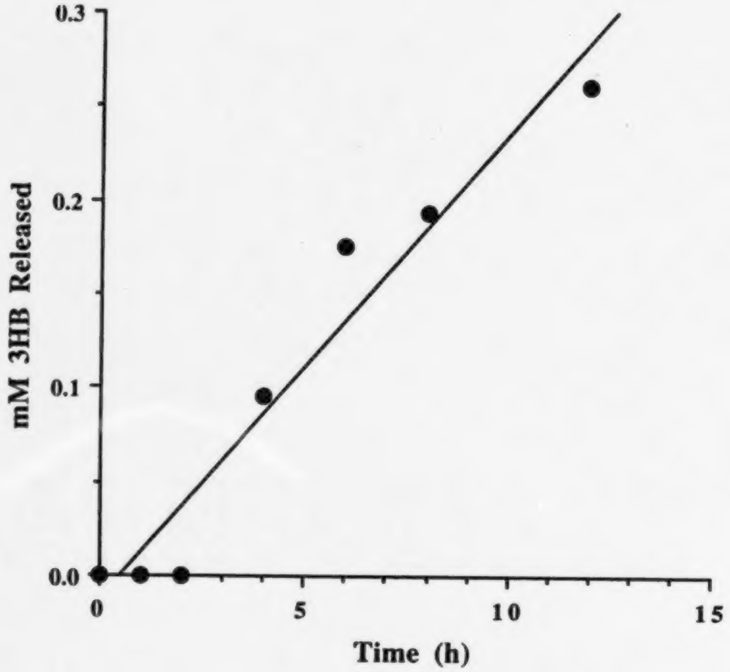


Figure 6.4. Depolymerase activity associated with P(3HB-co-15%3HV) granules removed from a 24-h-old *Staphylococcus* sp culture.

the depolymerase being impeded by crystalline areas on the granules (Nishida and Tokiwa, 1993). Presumably, the depolymerase will rapidly hydrolyze accessible ester bonds in amorphous regions on the granule first, leaving the crystalline, less accessible areas behind.

6.5 Summary and Conclusions

A facultative anaerobic bacterium isolated from rumen fluid was capable of biodegrading PHAs in liquid cultures under aerobic conditions. Exponential growth on PHAs occurred within about 36 h but cells seemed to remain viable and still able to utilize PHAs after 48 h since 2-week-old cultures of *Staphylococcus* sp. growing on PHAs (PHA granules in the culture medium were visible) were still viable. Depolymerase activity was only detected in PHA-grown cultures and not in glucose-grown cultures. The depolymerase may, therefore, be inducible. The depolymerase adsorbs to the granules and is active during and after the exponential growth phase.

A considerable amount of time and energy was spent on obtaining a reliable and reproducible assay system for measuring depolymerase activity. In order for the turbidimetric assay to be successful, the suspension of PHA granules had to be stable. A decrease in turbidity was supposed to signify that the PHA granules were being solubilized by the breakdown of the polymer chains into its monomeric units through the action of the depolymerase. However, in enzyme-free assay systems the granules would inevitably settle to the bottom of the assay tube causing a decrease in turbidity often detectable within 60 min. This was not long enough to detect a change in turbidity due to depolymerase activity.

The turbidimetric assay was modified such that the reaction tubes could be incubated for longer than 60 min and could be mixed to resuspend any settled granules. Theoretically, the absorbance of enzyme-free assay systems should remain the same after long periods of incubation and mixing to resuspend granules. However, in practice the absorbance readings in enzyme-free assay systems as well as the test assay systems were very scattered. It was very likely that the granules were of uneven size and despite extensive sonication did not produce a fine suspension. Resuspending and mixing the granules probably caused the distribution of the different-sized granules to change resulting in fluctuations of absorbance measurements.

Other factors that could have contributed to the failure of obtaining a reliable turbidimetric depolymerase assay included not knowing the pH and temperature optima of the depolymerase from *Staphylococcus* sp. A pH range of 7.5 to 9.5 was examined but results were inconclusive. A pH of 7.5 or 8.0 was normally chosen as this was the most cited pH used for turbidimetric depolymerase assays in the literature. But the *Staphylococcus* sp. depolymerase may not have been very active at these two pH values even though the pH optimum for cell growth was 7.4. Likewise, the optimum temperature for *Staphylococcus* sp. depolymerase was not known. The assays were typically run at room temperature or at 30°C. The depolymerase may have been more active at higher temperatures and so a change in turbidity might have been detected.

Another important factor that probably contributed to the failure of the assay systems was the uncertainty of the amount of crude enzyme added to the reaction tubes. The amount of depolymerase present in the culture supernatants could have been very small and therefore difficult to detect. However, an attempt to detect depolymerase activity from a sample of concentrated culture supernatant did not work. The depolymerase may have been denatured or lost during the concentration procedure or it may simply not have been present in the supernatant to begin with. It was discovered later when the depolymerase assay of Janssen and Schink (1993) was tried, that the majority of the depolymerase activity was associated with the PHA granules. Little or no activity was detected in the culture supernatant by this method. The depolymerase assay of Janssen and Schink (1993) was found to be the best method for measuring depolymerase activity. However, the analytical techniques involved with this method potentially gave rise to many sources of errors. The presence of any PHA granules in the methanolysis reaction gave rise to elevated 3HB concentrations. As well, some 3HB could have been lost during the extraction procedure after the methanolysis reaction was finished. Nevertheless, this assay system was very reproducible.

7. OVERALL SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH

Studies described in the Introduction and Literature Review chapter showed that PHAs are biodegradable in a variety of aerobic environments by a large number of different bacteria and fungi. Indications that PHAs could be biodegraded under anaerobic conditions was suggested by the isolation of an anaerobic PHA-degrading bacterium from sediment (Janssen and Harfoot, 1990).

This study examined the fate of PHAs under different anaerobic conditions. Mixed and pure cultures were used as sources of degrading bacteria. Results showed that PHAs were biodegraded under nitrate-reducing conditions with an activated sludge consortium. Nitrate was primarily reduced to nitrous oxide. Nitrogen formed only when the nitrate levels in the cultures were very low. The amount of PHAs decreased by 20% from the original amount added to cultures within a 40 day period.

The biodegradation of PHAs coupled to ferric iron- and sulfate-reduction could not be demonstrated. There were large variations in data most likely arising from uneven distribution of bacteria amongst the cultures. As well, conflicting results were obtained with mixed cultures under iron-reducing conditions. Cultures showing enhanced metabolic activity as indicated by high CO_2 production did not have a corresponding high Fe(III)-reduction. However, cultures with low metabolic activity did have enhanced Fe(III)-reduction occurring. The pH within these cultures also changed over the course of the incubation period.

Of the methanogenic environments tested, sewage sludge was the most amenable to the biodegradation of PHAs. The total amounts of gas produced from the biodegradation of PHAs were between 83 to 96% of the theoretical amounts. The hydrolysis of PHAs was shown to be the rate-limiting step in the methanogenic degradation of PHAs, however the overall process occurred quite rapidly as significant biodegradation of PHAs occurred within 3 days. An anaerobic digestor is known to contain a large and diverse microbial population. The number of bacteria in a typical digestor is estimated to be between 10^6 to 10^{10} organisms in one millilter of sludge and over 19 genera and 50 species of non-methanogenic bacteria have been detected (Young, 1984). Since the organic matter load on a digestor is so high, the sludge bacteria out of necessity, are capable of degrading lipids, cellulose, proteins, carbohydrates and other complex polymers at a fast rate. Fermentative bacteria in the rumen, on the other hand, are exposed to a more limited range of polymers such as cellulose, starch and pectin (Young, 1984). However, this does not preclude that rumen microorganisms are capable of degrading PHAs since a *Staphylococcus* species capable of utilizing PHAs was isolated from rumen fluid. Janssen and Harfoot (1990) were able to isolate an anaerobic bacterium, *I. delafieldii*, from sediment that was capable of degrading P(3HB). Results in this thesis showed that a sediment consortium was able to biodegrade PHAs under methanogenic conditions. Organisms similar to *I. delafieldii*, in terms of hydrolyzing capabilities of PHAs, were most likely present in the sediment.

The anaerobic biodegradation of PHAs appears to basically use the same strategy as aerobic biodegradation does, namely the breakdown of PHA into its monomeric units with their subsequent absorption into bacterial cells. *I. delafieldii* ferments 3HB to acetate and butyrate (Janssen and Schink, 1993) The complete biodegradation of PHAs under anaerobic conditions seems to require a consortium of microorganisms cooperating together. The fermentative bacteria break PHA down and microorganisms using terminal electron acceptors oxidize the resulting fermentative endproducts.

The isolated *Staphylococcus* sp. mentioned above was shown to be capable of growing on PHAs both aerobically and anaerobically. Attempts were made to measure depolymerase activity in culture supernatant using assays reported in the literature. However, unreliable results were obtained, mainly due to inherent problems with the actual assay. Depolymerase activity was demonstrated with an assay system involving the detection of 3HB formation from the degradation of PHA by the depolymerase. Growth occurred within 36 h in liquid cultures of *Staphylococcus* sp. with PHAs as sole carbon sources. 3HB was detected in culture supernatant during early exponential phase and amounts began to decrease by stationary phase. Depolymerase activity was shown to be active within 12 h, after which the activity began to level off. This was presumably due to regions of highly crystalline areas on the granules retarding depolymerase activity.

Further characterization of the depolymerase from *Staphylococcus* sp. could be done. Ideally the depolymerase should be purified using methods described in the literature, for example those by Tanio *et al.* (1982), Shirmer *et al.* (1993) and Jendrossek *et al.* (1993). Better results may be obtained with the turbidimetric assays by using purified enzyme. The amount of enzyme added would be known and any possible inhibitors in the culture medium would have been removed. Substrate specificity, pH and temperature optima could be determined.

A reanalysis of the media used and environments sampled for the ferric iron-reduction experiments should be done. The buffering capacity of the media to prevent wide pH fluctuations should be optimized. Cultures set up with sediments from spring water should provide a good diversity and number of fermentative and ferric iron-reducing bacteria. As well cocultures of *Staphylococcus* sp. with *Shewanella putrefaciens* under iron-reducing conditions or with *Desulfovibrio sapovorans, Desulfococcus multivorans* or *Desulfosarcina variabilis* under sulfate-reducing conditions with PHAs as carbon substrates could be set up. Janssen and Schink (1993) were able to produce cocultures of *I. delafieldii* and *D. vulgaris* growing on P(3HB).

Methanogenic degradation of PHAs was demonstrated using amorphous PHA granules. However, in commercial applications, PHAs are formed, for example, into films or bottles. Experiments could be set up looking at the rate of biodegradation of PHA films with sewage sludge as the inoculum, to see how this compares with the rates obtained from using granules. Sludge cultures could be set up in the laboratory or *in situ* tests could be performed. This should give a grasp of the actual lifespan of PHA materials under methanogenic conditions.

8. LITERATURE CITED

AMOS, D., and M.J. McINERNEY. 1991. Composition of poly-ß-hydroxyalkanoate from *Syntrophomonas wolfei* grown on unsaturated fatty acid substrates. Arch. Microbiol. **155**:103-106.

ANDERSON, A.J. and E.A. DAWES. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54**:450-472.

ASTM. 1992. D638-91. Standard test method for tensile properties of plastics, p. 159-171. *In* Annual book of ASTM standards, vol. 08.01. ASTM, Philadelphia.

ASTM. 1992. D882-91. Standard test method for tensile properties of plastics of thin plastic sheeting, p. 313-321. *In* Annual book of ASTM standards, vol. 08.01. ASTM, Philadelphia.

BANAT, I.M., D.B. NEDWELL and M. T. BALBA. 1983. Stimulation of methanogenesis by slurries of saltmarsh sediment after the addition of molybdate to inhibit sulphate-reducing bacteria. J. Gen. Microbiol. **129**:123-129.

BERGER, E., B.A. RAMSAY, J.A. RAMSAY, and C. CHAVARIE. 1989. PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnol. Tech. **3**:227-232.

BERNARD, G.N., and J.K.M. SANDERS. 1989. The poly-ß-hydroxybutyrate granule *in vivo*. A new insight based on NMR spectroscopy of whole cells. J. Biol. Chem. **264**:3286-3291.

BIEDERMANN, J., M. STANISZEWSKI, S. WAIS and R. SUESSMUTH. 1993. Poly-ß-hydroxybutyrate/ß-hydroxyvalerate-copolymers as a substrate and a matrix for microorganisms in denitrification of drinking water, p. 473-474. *In* H.G. Schlegel and A. Steinbüchel (ed.), Proceedings of the International Symposium on Bacterial Polyhydroxyalkanoates. Goltze-Druck, Göttingen, Germany.

BLOEMBERGEN, S., D.A. HOLDEN, G.K. HAMER, T.L. BLUHM and R.H. MARCHESSAULT. 1986. Studies of composition and crystallinity of bacterial poly(ß-hydroxybutyrate-co-ß-hydroxyvalerate). Macromolecules. **19**:2865-2871.

BONTHRONE, K.M., J. CLAUSS, D.M. HOROWITZ, B.K. HUNTER and J.K.M. SANDERS. 1992. The biological and physical chemistry of polyhydroxyalkanoates as seen by NMR spectroscopy. FEMS Microbiol. Rev. **103**:269-278.

BOONE, D.R. 1991. Ecology of methanogenesis, p. 57-69. *In* J.E. Rogers and W.B. Whitman. (ed.), Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides, and Halomethanes. American Society for Microbiology. Washington D.C.

BOSSERT, I.D., M.D. RIVERA and L.Y. YOUNG. 1986. p-Cresol biodegradation under denitrifying conditions: isolation of a bacterial coculture. FEMS Microbiol. Ecology. **38**:313-319.

BOUWER, E.J. and P.L. McCARTY. 1983. Effects of 2-bromoethanesulfonic acid and 2-chloroethanesulfonic acid on acetate utilization in a continuous-flow methanogenic fixed-film column. Appl. Environ. Microbiol. **45**:1408-1410.

BRANDL, H. and P. PÜCHNER. 1990. The degradation of shampoo bottles in a lake, p. 421-422. *In* E.A. Dawes (ed.), Novel Biodegradable Microbial Polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.

BRANDL, H., R.A. GROSS, R.W. LENZ, and R.C. FULLER. 1988. *Pseudomonas oleovorans* as a source of poly(ß-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54**:1977-1982.

BRANDL, H., R.A. GROSS, R.W. LENZ, and R.C. FULLER. 1990. Plastics from bacteria and for bacteria: poly(ß-hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters. Adv. Biochem. Eng. Biotechnol. **41**;77-93.

BRAUN, K. and D.T. GIBSON. 1984. Anaerobic degradation of 2-aminobenzoate (anthranilic acid) by denitrifying bacteria. Appl. Environ. Microbiol. **48**:102-107.

BRIESE, B.H., B. SCHMIDT and D. JENDROSSEK. 1994. *Pseudomonas lemoignei* has five poly(hydroxyalkanoic acid) (PHA) depolymerase genes: a comparative study of bacterial and eukaryotic PHA depolymerases. J. Environ. Polymer Degrad. **2**:75-87.

BRIESE, B.H., D. JENDROSSEK and H.G. SCHLEGEL. 1994. Degradation of poly(3hydroxybutyrate-co-3hydroxyvalerate) by aerobic sewage sludge. FEMS. Microbiol. Lett. **117**:107-112.

BRUCATO, C.L. and S.S. WONG, 1991. Extracellular poly(3-hydroxybutyrate) depolymerase from *Penicillium funiculosum*: general characteristics and active site studies. Arch. Biochem. Biophys. **290**:497-502.

BRYANT, M.P. 1979. Microbial methane production-theoretical aspects. J. Animal Sci. **48**:193-201.

BUSWELL, A.M. and H.F. MÜLLER. 1952. Mechanism of methane fermentation. Ind. Eng. Chem. **44**:550-552.

CHOWDHURY, A.A. 1963. Poly-ß-hydroxybuttersaure abbauende bakterien und exoenzym. Arch. Mikrobiol. **47**:167-200.

COLLINS, M.D. and F. WIDDEL. 1986. Respiratory quinones of sulphate-reducing and sulphur-reducing bacteria: A systematic investigation. System. Appl. Microbiol. **8**:8-18.

COLLINS, S. 1987. Choice of substrate in polyhydroxybutyrate synthesis. Spec. Publ. Soc. General Microbiol. **21**:161-168.

CORNIBERT, J., and R.H. MARCHESSAULT. 1972. Physical properties of poly-ß-hydroxybutyrate. IV. Conformational analysis and crystalline structure. J. Mol. Biol. **71**:735-756.

DAVIDSON, E.A. 1991. Fluxes of nitrous oxide and nitric oxide from terrestrial ecosystems, p. 219-235. *In* J.E. Rogers and W.B. Whitman. (ed.), Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides, and Halomethanes. American Society for Microbiology Press, Washington D.C.

DAWES, E.A. and P J. SENIOR. 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **10**:135-266.

DE SMET, M.J., G. EGGINK, B. WITHOLT, J. KINGMA and H. WYNBERG. 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. **154**:870-878.

DELAFIELD, F.P., K.E. COOKSEY, and M. DOUDOROFF. 1965a. ß-hydroxybutyric dehydrogenase and dimer hydrolase of *Pseudomonas*

DELAFIELD, F.P., M. DOUDOROFF, N.J. PALLERONI, C.J. LUSTY and R. CONTOPOULOS. 1965b. Decomposition of poly-ß-hydroxybutyrate by pseudomonads. J. Bacteriol. **90**:1455-1466.

DiCHRISTINA, T.J. and E.F. DeLONG. 1994. Isolation of anaerobic respiratory mutants of *Shewanella putrefaciens* and genetic analysis of mutants deficient in anaerobic growth on Fe^{3+}. J. Bacteriol. **176**:1468-1474.

DOI, Y. 1990. Microbial Polyesters. VCH Publishers, Inc., New York.

DOI, Y., A. SEGAWA, Y. KAWAGUCHI and M. KUNIOKA. 1990a. Cyclic nature of poly(3-hydroxyalkanoate) metabolism in *Alcaligenes eutrophus*. FEMS Microbiol. Lett. **67**:165-170.

DOI, Y., Y. KANESAWA and M. KUNIOKA. 1990b. Biodegradation of microbial copolyesters: poly(3-hydroxybutyrate-co-3-hyroxyvalerate) and poly(3-hydroxybutyrate-co-4-hyroxybutyrate). Macromolecules **23**:26-31.

DOI, Y., Y. KANESAWA, N. TANAHASHI and Y. KUMAGAI. 1992. Biodegradation of microbial polyesters in the marine environment. Polym. Degrad. Stabil. **36**:173-177.

DOI, Y., Y. KAWAGUCHI, Y. NAKAMURA and M. KUNIOKA. 1989. Nuclear magnetic resonance studies of poly(3-hydroxybutyrate) and polyphosphate metabolism in *Alcaligenes eutrophus*. Appl. Environ. Microbiol. **55**:2932-2938.

DUNNETT, C.W. 1955. A multiple comparison procedure for comparing several treatments with a control. Am. Stat. Assoc. J. **50**:1096-1121.

EASTMAN, J.A. and J.F. FERGUSON. 1981. Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. J. Water Pollut. Control Fed. **53**:352-366.

EDWARDS, E., 1994. Laboratory search for microorganisms capable of degrading benzene under anaerobic conditions, p.435-439. *In* Proceedings of the Fourth Annual Symposium on Groundwater and Soil Remediation. Calgary, Alberta Sept. 21-23.

FEDORAK, P.M. and D. GRBIC'-GALIC'. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. **57**:932-940.

FEDORAK, P.M. and S.E. HRUDEY. 1983. A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. Environ. Technol. Lett. **4**:425-432.

FEDORAK, P.M. and S.E. HRUDEY. 1984. The effects of phenol and some alkyl phenolics on batch anaerobic methanogenesis. Water Res. **18**:361-367.

FEDORAK, P.M., J.M. FOGHT and D.W.S. WESTLAKE. 1982. A method for monitoring mineralization of ^{14}C-labeled compounds in aqueous samples. Water Res. **16**:1285-1290.

FEDORAK, P.M., K.M. SEMPLE and D.W.S. WESTLAKE. 1987. A statistical comparison of two culturing methods for enumerating sulfate-reducing bacteria. J. Microbiol. Methods. **7**:19-27.

FERRY, J.G. 1992. Methane from acetate. J. Bacteriol. **174**:5489-5495.

FINDLAY, R.H. and D.C. WHITE. 1983. Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. Appl. Environ. Micobiol. **45**:71-78.

FUKUI, T., T. NARIKAWA, K. MIWA, Y. SHIRAKURA, T. SAITO and K. TOMITA. 1988. Effect of limited tryptic modification of a bacterial poly(3-hydroxybutyrate) depolymerase on its catalytic activity. Biochim. Biophys. Acta. **952**:164-171.

FUKUI, T., T. YOSHIMOTO, M. MATSUMOTO, S. HOSKAWA, T. SAITO, H. NISHIKAWA and K. TOMITA. 1976. Enzymatic synthesis of poly-ß-hydroxybutyrate in *Zoogloea ramigera*. Arch. Microbiol. **110**:149-1156.

GALVIN, T.J. 1990. PHBV^{TM} biodegradable polyester, p. 39-43. *In* S.A. Barenberg, J.L. Brash, R. Narayan and A.E. Redpath (ed.), Degradable Materials: Perspectives, Issues and Opportunities. CRC Press Inc. Boca Raton, FL.

GARCIA, J.L. 1990. Taxonomy and ecology of methanogens. FEMS Microbiol. Lett. **87**:297-308.

GILMORE, D.F., R.C. FULLER and R. LENZ. 1990. Biodegradation of poly(beta-hydroxyalkanoates), p. 481-507. *In* S.A. Barenberg, J.L. Brash, R. Narayan and A.E. Redpath (ed.), Degradable Materials: Perspectives, Issues and Opportunities. CRC Press Inc. Boca Raton, FL.

GILMORE, D.F., R.C. FULLER, B. SCHNEIDER, R.W. LENZ, N. LOTTI and M. SCANDOLA. 1994. Biodegradabilty of blends of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with cellulose acetate esters in activated sludge. J. Environ. Polym. Degrad. **1**:49-57.

GILMORE, D.F., S. ANTOUN, R.W. LENZ, S. GOODWIN, R. AUSTIN and R.C. FULLER. 1992. The fate of 'biodegradable' plastics in municipal leaf compost. J. Indust. Microbiol. **10**:199-206.

GRIEBEL, R. and J.M. MERRICK. 1971. Metabolism of poly-ß-hydroxybutyrate: effect of mild alkaline extraction on native poly-ß-hydroxybutyrate granules. J. Bacteriol. **108**:782-789.

GRIEBEL, R., Z. SMITH and J.M. MERRICK. 1968. Metabolism of poly-ß-hydroxybutyrate. I. Purification, composition and properties of native poly-ß-hydroxybutyrate granules from *Bacillus megaterium*. Biochemistry **7**:3676-3681.

GROSS, R.A., C. DEMELLO, R.W. LENZ, H. BRANDL and R.C. FULLER. 1989. Biosynthesis and characterization of poly(ß-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. Macromolecules. **22**:1106-1115.

GUIRARD, B.M. and E.E. SNELL. 1981. Biochemical factors in growth, p. 95. *In* P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Aood, N.R. Krieg and G.B. Phillips (ed.), Manual of Methods for General Bacteriology. American Society for Microbiology. Washington D.C.

GUJER, W. and A.J.B. ZEHNDER. 1983. Conversion processes in anaerobic digestion. Water Sci. Technol. **15**:127-167.

HARRISON, S.T.L., H.A. CHASE and J.S. DENNIS. 1991. The lysis of Gram-negative *Alcaligenes eutrophus* by enzymes from *Cytophaga*. Biotech. Tech. **5**:115-120.

HAYWOOD, G.W., A.J. ANDERSON, and E.A. DAWES. 1989. A survey of the accumulation of novel polyhydroxyalkanoates by bacteria. Biotechnol. Lett. **11**:471-476.

HOLLAND, S.J., A.M. JOLLY, M. YASHIN AND B.J. TIGHE. 1987. Polymers for biodegradable medical devices. Biomaterials. **8**:289-295.

HOLMES, P.A. 1985. Applications of PHB-a microbially produced biodegradable thermoplastic. Phys. Technol. **16**:32-36.

HOLMES, P.A. and G.B. LIM. March 1990. Separation process. U.S. patent 4,910,145.

HUNGATE, R.E. 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. *In* J.R. Norris and D.W. Ribbons (ed.), Methods in Microbiology, vol. 3B. Academic Press Inc., New York.

JACKSON, F.A. and E.A. DAWES. 1976. Regulation of the tricarboxylic acid cycle and poly-ß-hydroxybutyrate metabolism in *Azotobacter beijerinckii* grown under nitrogen or oxygen limitation. J. Gen. Microbiol. **97**:303-312.

JANSSEN, P.H. and B. SCHINK. 1993. Pathway of anaerobic poly-ß-hydroxybutyrate degradation by *Ilyobacter delafieldii*. Biodegradation **4**:179-185.

JANSSEN, P.H. and B. SCHINK. 1993. Pathway of anaerobic poly-ß-hydroxybutyrate degradation by *Ilyobacter delafieldii*. Biodegradation **4**:179-185.

JANSSEN, P.H. and C.G. HARFOOT. 1990. *Ilyobacter delafieldii* sp. nov., a metabolically restricted anaerobic bacterium fermenting PHB. Arch. Microbiol. **154**:253-259.

JENDROSSEK, D., I. KNOKE, R.B. HABIBIAN, A. STEINBÜCHEL and H. G. SCHLEGEL. 1993. Degradation of poly(3-hydroxybutyric acid), PHB, by bacteria and purification of a novel PHB depolymerase from *Comamonas* sp. J. Environ. Polym. Degrad. **1**:53-63.

JESUDASON, J.J., R.H. MARCHESSAULT and T. SAITO. 1993. Enzymatic degradation of poly([R,S] ß-hydroxybutyrate). J. Environ. Polym. Degrad. **1**:89-98.

KUMAGAI, Y., and Y. DOI. 1993. Synthesis of a block copolymer of poly(3-hydroxybutyrate) and poly(ethylene glycol) and its application to biodegradable polymer blends. J. Environ. Polym Degrad. **1**:81-87.

KUMAGAI, Y., Y. KANESAWA and Y. DOI. 1992. Enzymatic degradation of microbial poly(3-hydroxybutyrate). Makromol. Chem. **193**:53-57.

LAUZIER, C., J.-F. REVOL and R.H. MARCHESSAULT. 1992. Topotactic crystallization of isolated poly(ß-hydroxybutyrate) granules from *Alcaligenes eutrophus*. FEMS Microbiol. Rev. **103**:299-310.

LAW, J.H. and R.A. SLEPECKY. 1961. Assay of poly-ß-hydroxybutyrate acid. J. Bacteriol. **82**:33-36.

LIEBERGESELL, M., E. HUSTEDE, A. TIMM, A. STEINBÜCHEL, R.C. FULLER, R.W. LENZ and H.G. SCHLEGEL. 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. Arch. Microbiol. **155**:415-421.

LOGSDON, G. 1989. Biodegradable breakthrough. Totally natural plastic. BioCycle. **30**(3):58-60.

LOPEZ-LLORCA, L., M.F. COLOM VALIENTE and A. GASCON. 1993. A study of polyhydroxyalkanoate (PHA) films in soil by scanning electron microscopy. Micron. **24**:23-29

LOPEZ-LLORCA, L., M.F. COLOM VALIENTE and M.J. CARCASES. 1994. Study of biofouling of polyhydroxyalkanoate (PHA) films in water by scanning electron microscopy. Micron. **25**:45-51

LOVLEY, D.R. 1991. Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Reviews **55**:259-287.

LOVLEY, D.R. and E.J.P. PHILLIPS. 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. **51**:683-689.

LOVLEY, D.R. and S. GOODWIN. 1988. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochimica. Cosmochimica. Acta. **52**:2993-3003.

LOVLEY, D.R., E.J.P. PHILLIPS and D.J. LONERGAN. 1989. Hydrogen and formate oxidation coupled to dissimilatory reduction of iron or manganese by *Alteromonas putrefaciens*. Appl. Environ. Microbiol. **55**:700-706.

LOVLEY, D.R., F.H. CHAPELLE and J.C. WOODWARD. 1994. Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. Environ. Sci. Technol. **28**:1205-1210.

LOVLEY, D.R., S.J. GIOVANNONI, D.C. WHITE, J.E. CHAMPINE, E.J.P. PHILLIPS, Y.A. GORBY and S. GOODWIN. 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch. Microbiol. **159**:336-344.

LOWRY, O.H., R.J. ROSEBROUGH, A.L. FARR, and R.J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.

LUSTY, C.J. and M. DOUDOROFF. 1966. ß-Hydroxybutyrate depolymerase of *Pseudomonas lemoignei*. Proc. Natl. Acad. Sci. **56**:960-965.

LUZIER, W. 1992. Materials derived from biomasss/biodegradable materials. Proc. Natl. Acad. Sci. USA. **89**:839-842.

MACKIE, R.I, B.A. WHITE and M.P. BRYANT. 1991. Lipid metabolism in anaerobic ecosystems. Crit. Rev. Microbiol. **17**:449-479.

MANCHAK, J. and W.J. PAGE. 1994. Control of poly-ß-hydroxyalkanoate synthesis in *Azotobacter vinelandii* strain UWD. Microbiol. **140**:953-963.

MARCHESSAULT, R.H., C.J. MONASTERIOS and P. LEPOUTRE. 1990. Properties of poly-ß-hydroxyalkanoate latex: nascent morphology, film formation and surface chemistry, p. 97-112. *In* E.A. Dawes (ed.), Novel Biodegradable Microbial Polyesters. Kluwer Academic Publishers, Dordrecht, The Netherlands.

MATAVULJ, M. and H.P. MOLITORIS. 1992. Fungal degradation of polyhydroxyalkanoates and a semiquantitative assay for screening their degradation by terrestrial fungi. FEMS Microbiol. Rev. **103**:323-332.

MATAVULJ, M., S.T. MOSS and H.P. MOLITORIS. 1993. Degradation of poly-ß-hydroxyalkanoate-based plastics in natural environments, p. 465-466. *In* H.G. Schlegel and A. Steinbüchel (ed.), Proceedings of the International Symposium on Bacterial Polyhydroxyalkanoates. Goltze-Druck, Göttingen, Germany.

McINERNEY, M.J., D.A. AMOS, K.S. KEALY and J.A. PALMER. 1992. Synthesis and function of polyhydroxyalkanoates in anaerobic syntrophic bacteria. FEMS Microbiol. Rev. **103**:195-206.

McINERNEY, M.J., D.A. AMOS, K.S. KEALY and J.A. PALMER. 1992. Synthesis and function of polyhydroxyalkanoates in anaerobic syntrophic bacteria. FEMS Microbiol. Rev. **103**:195-206.

McINERNEY, M.J., M.P. BRYANT and N. PFENNIG. 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. **122**:129-135.

McLELLAN, D.W. and P.J. HALLING. 1988. Acid-tolerant poly(3-hydroxybutyrate) hydrolases from moulds. FEMS Microbiol. Lett. **52**:215-218.

MERGAERT, J., A. WEBB, C. ANDERSON, A. WOUTERS and J. SWINGS. 1993. Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3 hydroxyvalerate) in soils. Appl. Environ. Microbiol. **59**:3233-3238.

MERGAERT, J., C. ANDERSON, A. WOUTERS, J. SWINGS and K. KERSTERS. 1992. Biodegradation of polyhydroxyalkanoates. FEMS Microbiol. Rev. **103**:317-322.

MERRICK, J. M. and M. DOUDOROFF. 1964. Depolymerization of poly-ß-hydroxybutyrate by an intracellular enzyme system. J. Bacteriol. **88**:60-71.

MILLER, T.L. and M.J. WOLIN. 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27**:985-987.

MORRIS, C. (ed.). 1992. Academic Press Dictionary of Science and Technology. Academic Press, Harcourt Brace Jovanovich, Publishers. San Diego.

MOSKOWITZ, G.J. and J.M. MERRICK. 1969. Metabolism of poly-ß-hydroxybutyrate. II. Enzymatic synthesis of D-(-)-ß-hydroxybutyryl coenzyme A by an enoyl hydrase from *Rhodospirillum rubrum*. Biochemistry **8**:2748-2755.

MUKAI, K., K. YAMADA and Y. DOI. 1992. Extracellular poly(hydroxyalkanoate) depolymerases and their inhibitor from *Pseudomonas lemoignei*. Int. J. Biol, Macromol. **14**:235-239.

MUKAI, K., K. YAMADA and Y. DOI. 1993a. Kinetics and mechanism of heterogeneous hydrolysis of poly[(R)-3-hydroxybutyrate] film by PHA depolymerases. Int. J. Biol. Macromol. **15**:361-366.

MUKAI, K., K. YAMADA and Y. DOI. 1993b. Enzymatic degradation of poly(hydroxyalkanoates) by a marine bacterium. Polym. Degrad. Stabil. **41**:85-91.

MUKAI, K., Y. DOI, Y. SEMA and K. TOMITA. 1993c. Substrate specificities in hydrolysis of polyhydroxyalkanoates by microbial esterases. Biotech. Lett. **15**:601-604.

MÜLLER, B. and D. JENDROSSEK. 1993. Purification and properties of poly(3-hydroxyvaleric acid) depolymerase from *Pseudomonas lemoignei*. Appl. Microbiol. Biotechnol. **38**:487-492.

NAKAYAMA, K., T. SAITO, T. FUKUI, Y. SHIRAKURA and K. TOMITA. 1985. Purification and properties of extracellular poly(3-hydroxybutyrate) depolymerase from *Pseudomonas lemoignei*. Biochim. Biophys. Acta. **827**:63-72.

NICHOLSON, J.W. 1991. Mechanical properties of polymers, p. 104-129. *In* The Chemistry of Polymers. The Royal Society of Chemistry, Thomas Graham House, Cambridge.

NISHIDA, H. and Y. TOKIWA. 1993. Effect of higher-order structure of poly(3-hydroxybutyrate) on its biodegradation. II. Effects of crystal structure on microbial degradation. J. Environ. Polym. Degrad. **1**:65-80.

OBUEKWE, C.O., WESTKAKE, D.W.S., and F.D. COOK. 1981. Effect of nitrate on reduction of ferric iron by a bacterium isolated from crude oil. Can. J. Microbiol. **27**:692-697.

OEDING, V. and H.G. SCHLEGEL. 1973. ß-ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-ß-hydroxybutyrate metabolism. Biochem. J. **134**:239-248.

ORTS, W.J., R.H. MARCHESSAULT, T.L. BLUHM and G.K. HAMER. 1990. Observation of strain-induced ß form in poly(ß-hydroxyalkanoates). Macromolecules. **23**:5368-5370.

PAGE, W.J. 1989. Production of poly-ß-hydroxybutyrate by *Azotobacter vinelandii* strain UWD during growth on molasses and other complex carbon sources. Appl. Microbiol. Biotechnol. **31**:329-333.

PAGE, W.J. 1992. Production of polyhydroxyalkanoates by *Azotobacter vinelandii* UWD in beet molasses culture. FEMS Microbiol. Rev. **103**:149-158.

PAGE, W.J. and A. CORNISH. 1993. Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of poly-ß-hydroxybutyrate. Appl. Microbiol. Biotechnol. **59**:4236-4244.

PAGE, W.J. and O. KNOSP. 1989. Hyperproduction of poly-ß-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD. Appl. Microbiol. Biotechnol. **55**:1334-1339.

PAGE, W.J., J. MANCHAK and B. RUDY. 1992. Formation of poly(hydroxybutyrate-co-hydroxyvalerate) by *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. **58**:2866-2873.

PALMISANO, A.C., D.A. MARUSCIK and B.S. SCHWAB. 1993. Enumeration of fermentative and hydrolytic micro-organisms from three sanitary landfills. J. Gen. Microbiol. **139**:387-3391.

PFEFFER, J.T. 1974. Temperature effects on anaerobic fermentation of domestic refuse. Biotechnol. Bioeng. **16**:771-787.

POIRIER, Y., D.E. DENNIS, K. KLOMPARENS and C. SOMERVILLE. 1992a. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science **256**:520-523.

POIRIER, Y., D.E. DENNIS, K. KLOMPARENS, C. NAWRATH and C. SOMERVILLE. 1992b. Perspectives on the production of polyhydroxyalkanoates in plants. FEMS Microbiol. Rev. **103**:237-246.

POSTGATE, J.R. 1979. The sulphate-reducing bacteria. Cambridge University Press, Cambridge.

PÜCHNER, P. and W.-R. MÜLLER. 1992. Aspects on biodegradation of PHA, p. 469-470. *In* H.G. Schlegel and A. Steinbüchel (ed.), Proceedings of the International Symposium on Bacterial Polyhydroxyalkanoates. Goltze-Druck, Göttingen, Germany.

RAMSAY, B.A., J.A. RAMSAY and D.G. COOPER. 1989. Production of poly-ß-hydroxyalkanoic acid by *Pseudomonas cepecia*. Appl. Environ. Microbiol. **55**:584-589.

RAMSAY, B.A., V. LANGLADE, P.J. CARREAU and J.A. RAMSAY. 1993. Biodegradabilty and mechanical properties of poly-(ß-hydroxybutyrate-co-ß-hydroxyvalerate)-starch blends. Appl. Environ. Microbiol. **59**:1242-1246.

RAMSAY, J.A., E. BERGER, B.A. RAMSAY and C. CHAVARIE. 1990. Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment. Biotechnol. Tech. **4**:221-226.

REUSCH, R.N. 1992. Biological complexes of poly-ß-hydroxybutyrate. FEMS Microbiol. Rev. **103**:119-130.

ROBERTS, D.J. 1989. Methanogenic metabolism of *m*-cresol. Ph.D. Thesis. University of Alberta, Edmonton, Alberta, Canada.

RODEN, E.E and D.R. LOVLEY. 1993. Dissimilatory Fe(III) reduction by the marine microorganism *Desulfuromonas acetoxidans*. Appl. Environ. Microbiol. **59**:734-742.

RODRIGUEZ-VALERA, F. and J.A.G. LILLO. 1992. Halobacteria as producers of polyhydroxyalkanoates. FEMS Microbiol. Rev. **103**:181-186.

ROGERS, J.K. 1992. Biopol resin debuts in blow molded bottle. Modern Plastics. **69**:17.

SAITO, T., A. IWATA and T. WATANABE. 1993. Molecular structure of extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis* T1. J. Environ. Polym. Degrad. **1**:99-105.

SAITO, T., K. SUZUKI, J. YAMAMOTO, T. FUKUI, K. MIWA, K. TOMITA, S. NAKANISHI, S. ODANI, J.I. SUZUKI and K. ISHIKAWA. 1989. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the gene for poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. J. Bacteriol. **171**:184-189.

SAITO, T., K. TOMITA, K. JUNI and K. OOBA. 1991. *In vivo* and *in vitro* degradation of poly(3-hydroxybutyrate) in rat. Biomaterials. **12**:309-312.

SCHIRMER, A., D. JENDROSSEK and H. G. SCHLEGEL. 1993. Degradation of poly(3-hydroxyoctanoic acid) [P(3HO)] by bacteria: purification and properties of a P(3HO) depolymerase from *Pseudomonas fluorescens* GK13. Appl. Environ. Microbiol. **59**:1220-1227.

SCHUBERT, P., A. STEINBÜCHEL and H.G. SCHLEGEL. 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-ß-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170**:5837-5847.

SEMPLE, K.M. and D.W.S. WESTLAKE. 1987. Characterization of iron-reducing *Alteromonas putrefaciens* strains from oil field fluids. Can. J. Microbiol. **33**:366-371.

SENIOR, P J. and E.A. DAWES. 1973. The regulation of ß-ketothiolase metabolism in *Azotobacter beijerinckii*. Biochem. J. **134**:225-238.

SENIOR, P.J. and E.A. DAWES. 1971. Poly-ß-hydroxybutyrate biosynthesis and the regulation of glucose metabolism in *Azotobacter beijerinckii*. Biochem. J. **125**:55-66.

SHELTON, D.R. and J.M. TIEDJE. 1984. General method for determining anaerobic biodegradation potential. Appl. Environ. Microbiol. **47**:850-857.

SHIRAKURA, Y., T. FUKUI, T. SAITO, Y. OKAMOTO, T. NARIKAWA, K. KOIDE, K. TOMITA, T. TAKEMASA and S. MASAMUNE. 1986. Degradation of poly(3-hydroxybutyrate) by poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis* T1. Biochim. Biophys. Acta. **880**:46-53.

SHIRAKURA, Y., T. FUKUI, T. TANIO, K. NAKAYAMA, R. MATSUNO and K. TOMITA. 1983. An extracellular D(-)-3-hydroxybutyrate oligomer hydrolase from *Alcaligenes faecalis*. Biochim. Biophys. Acta. **748**:331-339.

SINGLETON, Jr., R. 1993. The sulfate-reducing bacteria: an overview, p.1-20. *In* J.M. Odom and R. Singleton, Jr. (ed.), The Sulfate-Reducing Bacteria: Contemporary Perspectives. Springer-Verlag, New York.

SLATER, S.C., W.H. VOIGE and D.E. DENNIS. 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-ß-hydroxybutyrate biosynthetic pathway. J. Bacteriol. **170**:4431-4436.

STEAL, R.G.D. and J.H. TORRIE. 1980. Principles and procedures of statistics. A biometric approach, 2nd. ed. McGraw-Hill Book Co., New York.

STEINBÜCHEL, A. 1990. Polyhydroxyalkanoic acids, p. 125-213. *In* D. Byrom (ed.), Biomaterials: Novel Materials from Biological Sources. Stockton Press, Macmillan Publishers Ltd. and ICI Biological Products Business.

STIEB, M. and B. SCHINK. 1984. A new 3-hydroxybutyrate fermenting anaerobe, *Ilyobacter polytropus*, gen. nov. sp. nov., possessing various fermentation pathways. Arch. Microbiol. **140**:139-146.

STINSON, M.W. and J.M. MERRICK. 1974. Extracellular enzyme secretion by *Pseudomonas lemoignei*. J. Bacteriol. **119**:152-161.

SZEWCZYK, E. and J. MIKUCKI. 1989. Poly-ß-hydroxybutyric acid in staphylococci. FEMS Microbiol. Lett. **61**:279-284.

TAL, S. and Y. OKON. 1985. Production of the reserve material poly-ß-hydroxybutyrate and its function in *Azospirillum brasilense* Cd. Can. J. Microbiol. **31**:608-613.

TANIO, T., T. FUKUI, Y. SHIRAKURA, T. SAITO, K. TOMITA, T. KAHO and S. MASAMUNE. 1982. An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. Eur. J. Biochem. **124**:71-77.

THAUER, R.K., K. JUNGERMANN and K. DECKER. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100-180.

TIEDJE, J.M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179-244. *In* A.J.B. Zehnder (ed.), Biology of Anaerobic Microorganisms. John Wiley & Sons, Inc., New York.

WALLEN, L.L. and W.K. ROHWEDDER. 1974. Poly-ß-hydroxyalkanoate from activated sludge. Environ. Sci. Technol. **8**:576-579.

WIDDEL, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, p. 469-586. *In* A.J.B. Zehnder (ed.), Biology of Anaerobic Microorganisms. Wiley, New York.

WILLIAMSON, D.H. and J.F. WILKINSON. 1958. The isolation and estimation of the poly-ß-hydroxybutyrate inclusions of *Bacillus cereus*. J. Gen. Microbiol. **19**:198-209.

WOFFORD, N.Q., P.S. BEATY and M.J. McINERNEY. 1986. Preparation of cell-free extracts and the enzymes involved in fatty acid metabolism in *Syntrophomonas wolfei*. J. Bacteriol. **167**:179-185.

WOLIN, M.J. 1982. Hydrogen transfer in microbial communities, p. 323-356. *In* A.T. Bull and J.H. Slater (ed.), Microbial Interactions and Communities, vol. 1. Academic Press, Inc., New York.

WURMTHALER, J and W.-R. MÜLLER. 1993. Use of PHB/PHV in water treatment, p. 475-476. *In* H.G. Schlegel and A. Steinbüchel (ed.), Proceedings of the International Symposium on Bacterial Polyhydroxyalkanoates. Goltze-Druck, Göttingen, Germany.

YAMADA, K., K. MUKAI and Y. DOI. 1993. Enzymatic degradation of poly(hydroxyalkanoates) by *Pseudomonas pickettii*. Int. J. Biol. Macromol. **15**:215-220.

YOUNG, L.Y. 1984. Anaerobic degradation of aromatic compounds, p. 487-535. *In* D.T. Gibson (ed.), Microbial Degradation of Organic Compounds. Marcel Dekker, Inc. New York.

ZHANG, J., T. SAITO, A. ICHIKAWA and T. FUKUI. 1992. Secretion of poly(3-hydroxybutyrate) in *Alcaligenes faecalis* T1. Chem. Pharm. Bull. **40**:713-717.

Appendix 1. Media Used in Experiments

Appendix 1.1 Burk's Medium for PHA Production in *Azotobacter vinelandii* strain UWD (ATCC 53799) (Page *et al.*, 1992)

Component	Amount
$MgSO_4$	0.81 mM
$CaSO_4$	0.58 mM
Na_2MoO_4	1 µM
Potassium Phosphate Buffer	5 mM
Ferric Citrate	50 µM
Glucose (w/v)	3.0%
Ammonium Acetate	15 mM
Fish Peptone[a] (w/v)	0.1%

[a] Product no. H0100BT; Protan A/S, Drammen, Norway

Preparation:

The individual components are added from stock solutions to give the final concentration given in the table above. Adjust pH to 7.2. The medium is then dispensed into flasks and autoclaved to sterilize. Glucose is usually added from a sterile stock after the medium has been sterilized.

Appendix 1.2 Amended Nitrate Medium (ANM) (Bossert *et al.*, 1986)

Stock components used in the medium:

	Compound	g/L distilled water
Trace Metals Solution[a]	$CaCl_2 \cdot 2H_2O$	3.7
	H_3BO_3	2.5
	$MnCl_2$	0.87
	$FeCl_3$	0.65
	$ZnCl_2$	0.44
	$Na_2MoO_4 \cdot 2H_2O$	0.29
	$CoCl_2$	0.01
	$CuCl_2$	0.0001

	Compound	mg/L distilled water
Vitamin Solution[b]	Biotin	2.0
	Folic acid	2.0
	Pyridoxine Hydrochloride	10
	Riboflavin	5.0
	Thiamine	5.0
	Nicotinic acid	5.0
	Pantothenic acid	5.0
	Vitamin B_{12}	0.1
	p-Aminobenzoic acid	5.0

[a] Trace metals solution of Fedorak and Grbic′-Galic′ (1991).

[b] The stock vitamin solution was prepared as a 100x concentrated stock and filtered sterilized.

Final composition of ANM[c]:

Component	Amount
Na_2HPO_4	7.9 g
KH_2PO_4	1.5 g
NH_4Cl	0.3 g
KNO_3	3.0 g
$FeSO_4 \cdot 7H_2O$	25.0 mg
$CuSO_4 \cdot 5H_2O$	0.3 mg
$NiCl_2 \cdot 6H_2O$	0.8 mg
Trace Mineral Solution	15.0 mL
Vitamin Solution (at 10% v/v)	1 mL
Distilled water	984 mL

[c] Yeast extract omitted.

Preparation:

Mix all components and adjust pH to 6.9. Boil for 2 min and sparge medium and headspace with O_2-free N_2 or He. While sparging dispense medium into serum bottles observing strict anaerobic techniques. Autoclave to sterilize. Sterile, anaerobic carbon substrates as solutions are added prior to inoculation.

Appendix 1.3 Medium for the Cultivation of SRB (Collins and Widdel, 1986)

Component solutions used in the medium:

		Amount		
Solution 1	Component	Marine	Brackish	Fresh
	NaCl	20.0 g	7.0 g	1.0 g
	$MgCl_2 \cdot 6H_2O$	3.0 g	1.2 g	0.4 g
	Na_2SO_4	4.0 g	4.0 g	4.0 g
	KH_2PO_4	0.2 g	0.2 g	0.2 g
	KCl	0.3 g	0.3 g	0.3 g
	$CaCl_2 \cdot 2H_2O$	0.15 g	0.15 g	0.15 g
	NH_4Cl	0.3 g	0.3 g	0.3 g
	Resazurin (0.1 g/L)	10 mL	10 mL	10 mL
	Distilled water	960 mL	960 mL	960 mL

	Component	Amount
Solution 2 (Trace Elements)	HCl	4.5 mL
	$FeCl_2 \cdot 4H_2O$	1.5 g
	H_3BO_4	60 mg
	$MgCl_2 \cdot 6H_2O$	100 mg
	$CoCl_2 \cdot 6H_2O$	120 mg
	$ZnCl_2$	70 mg
	$NiCl_2 \cdot 6H_2O$	25 mg
	$CuCl_2 \cdot 2H_2O$	15 mg
	$Na_2MoO_4 \cdot 2H_2O$	25 mg
	Distilled water	995 mL

	Component	Amount
Solution 3 (Selenite Solution)	$Na_2SeO_3 \cdot 5H_2O$	3.0 mg
	NaOH	0.5 mg
	Distilled water	1000 mL
Solution 4 (Buffer)	$NaHCO_3$	8.4 g
	Distilled water	100 mL
Solution 5 (Growth Factors)	Biotin	10 mg
	4-Aminobenzoic acid	40 mg
	Thiamine hydrochloride	100 mg
	Viatmin B_{12}	50 mg
	Nicotinic acid	100 mg
	Distilled water	1000 mL
Solution 6 (Reducing Agent)	$Na_2S \cdot 9H_2O$	1.2 g
	Distilled water	10 mL
Final Composition of Medium	Solution 1	970 mL
	Solution 2	1.0 mL
	Solution 3	1.0 mL
	Solution 4	30 mL
	Solution 5	1.0 mL
	Solution 6	1.0 mL

Preparation:

Prepare Solution 1 choosing the amounts of NaCl and $MgCl_2 \cdot 6H_2O$ depending on degree of salinity required for organism. Autoclave medium and sparge with O_2-free N_2. Solutions 2 and 3 are prepared, autoclaved and added to Solution 1. Solutions 4 and 5 are filtered sterilized and added to Solution 1. Solution 4 must be sparged with N_2 and is prepared fresh each time. The completed medium is then dispensed into sterile serum bottles that have been sparged with N_2. The bottles are quickly sealed with sterile butyl rubber stoppers and crimped with aluminum caps. Solution 6 is prepared by dissolving $Na_2S \cdot 9H_2O$ in boiling water, sparging with N_2 and dispensing into Hungate tubes and autoclaving. Solution 6 is added with sterile syringe and needle that had been rinsed with O_2-free N_2. Substrates are added as sterile solutions with sterile syringe and needle that had been rinsed with O_2-free N_2 gas. If the medium is not reduced after inoculation and during incubation, a few drops of 5% (v/v) sodium dithionite is added until color of medium goes from pink to colorless.

Modifications from Collins and Widdel (1986) method:

1) $NaHCO_3$ autoclaved in closed bottle under a CO_2 gas phase
2) thiaminium dichloride = thiamine hydrochloride
3) Media were prepared in flasks covered with aluminum foil and not in serum bottles as in original paper

Appendix 1.4 SRB Enrichment Medium or Postgate C Medium (Postgate, 1979).

Compound	Amount (g/L Distilled Water)
KH_2PO_4	0.5
NH_4Cl	1.0
Na_2SO_4	4.5
$CaCl_2 \cdot 6H_2O$	0.06
$MgSO_4 \cdot 7H_2O$	0.06
Sodium Lactate	6.0
Yeast Extract	1.0
$FeSO_4 \cdot 7H_2O$	0.004
Sodium Citrate$\cdot 2H_2O$	0.3

Preparation:

Add ingredients to distilled water and adjust pH to 7.5. Boil for 2 min, while cooling sparge medium and headspace with O_2-free N_2 or He. Continue sparging and dispense medium into serum bottles observing strict anaerobic techniques. Autoclave to sterilize. Substrates are added as sterile anaerobic solutions just prior to inoculation.

Appendix 1.5 B10 Medium for *Shewanella putrefaciens* ATCC 8071

(Obuekwe *et al.*, 1981)

Component	Amount
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4{\cdot}7H_2O$	0.2 g
NaCl	0.2 to 10.0 g
$MnSO_4$, Na_2MoO_4	1 mL of a combined 0.1% solution[a]
$CaSO_4$ (saturated)	10 mL
Yeast Extract	5.0 g
Bactopetone	5.0 g
Ferric phosphate	4.7 g
Distilled water	990 mL

[a] 0.1 g each of $MnSO_4$ and Na_2MoO_4 into 100 mL distilled water.

Preparation:

Dissolve ferric phosphate and add remaining ingredients before adjusting pH to 7.2. Boil for 2 min, while cooling sparge medium and headspace with O_2-free N_2 or He. Continue sparging and dispense medium into serum bottles observing strict anaerobic techniques. Autoclave to sterilize. Substrates are added as sterile anaerobic solutions just prior to inoculation.

Appendix 1.6 Modified Anaerobic Citrate Medium for *Shewanella putrefaciens* ATCC 8071 (Lovley *et al.*, 1989 and Lovley and Phillips, 1988)

Component	Amount
Ferric citrate	13.g
$NaHCO_3$	2.8 g
KCl	0.11 g
NH_4Cl	1.7 g
$NaH_2PO_4 \cdot H_2O$	0.67 g
L-Arginine·HCl	0.027 g
L-Glutamine	0.027 g
DL-Serine	0.044 g
Wolfe's Vitamin Solution[a]	10 mL
Wolfe's Mineral Solution[a]	10 mL

[a] See Appendix 1.7 for composition.

Preparation:

Boil ferric citrate in distilled water to dissolve. Add remaining ingredients, adjust pH to 6.8 and bring volume to 1 L. Boil for 2 min, while cooling sparge medium and headspace with O_2-free N_2 or He. Continue sparging and dispense medium into serum bottles observing strict anaerobic techniques. Autoclave to sterilize. Substrates are added as sterile anaerobic solutions just prior to inoculation.

Appendix 1.7 Anaerobic Citrate Medium ATCC 1768 (for *Geobacter metallireducens* ATCC 53774)

Stock components used in the medium:

	Component	Amount
Wolfe's Vitamin Solution	Biotin	2.0 mg
	Folic Acid	2.0 mg
	Pyroxidine HCl	10.0 mg
	Thiamine HCl	5.0 mg
	Riboflavin	5.0 mg
	Nicotinic Acid	5.0 mg
	Na-Pantothenate	5.0 mg
	Cyanocobalamine	0.1 mg
	p-Aminobenzoic Acid	5.0 mg
	Thioctic Acid	5.0 mg
	Distilled Water	1.0 L
Wolfe's Mineral Solution	Nitrilotriacetic Acid	1.5 g
	$MgSO_4 \cdot 7H_2O$	3.0 g
	$MnSO_4 \cdot H_2O$	0.5 g
	NaCl	1.0 g
	$FeSO_4 \cdot 7H_2O$	0.1 g
	$CoCl_2 \cdot 6H_2O$	0.1 g
	$CaCl_2$	0.1 g
	$ZnSO_4 \cdot 7H_2O$	0.1 g
	$CuSO_4 \cdot 5H_2O$	0.01 g
	$AlK(SO_4)_2$	0.01 g
	H_3BO_3	0.01 g
	$Na_2MoO_4 \cdot 2H_2O$	0.01 g
	Distilled Water	1.0 L

Preparation of Wolfe's Mineral Solution:

Add nitrilotriacetic acid to approximately 500 mL of distilled water and adjust to pH 6.5 with 2M KOH. Then add the other ingredients in order they are listed and dissolve. Adjust the volume to 1.0 L.

	Component	Amount
Trace Element Solution	Wolfe's Mineral Solution	1.0 L
	$NiCl_2 \cdot 6H_2O$	24.0 mg

Final composition of Anaerobic Citrate Medium:

Component	Amount
Ferric citrate	12.50 g
$NaHCO_3$	2.5 g
KCl	0.1 g
NH_4Cl	1.5 g
$NaH_2PO_4 \cdot H_2O$	0.6 g
Wolfe's Vitamin Solution	10 mL
Trace Element Solution	10 mL

Preparation:

Boil ferric citrate in distilled water to dissolve. Add remaining ingredients, adjust pH to 7.0 and bring volume to 1.0 L. Boil for 2 min, while cooling sparge medium and headspace with O_2-free N_2 or He. Continue sparging and dispense medium into serum bottles observing strict anaerobic techniques. Autoclave to sterilize. Substrates are added as sterile anaerobic solutions just prior to inoculation.

Appendix 1.8 Ferric Iron Enrichment Medium (Roden and Lovley, 1993)[a]

Component	Amount
Ferric phosphate	3.0 g
$NaHCO_3$	2.5 g
NH_4Cl	1.5 g
KH_2PO_4	0.6 g
KCl	0.1 g
Trace metals[b]	1.0 mL
Distilled water	999 mL

[a] Modifications are the substitutions of ferric pyrophosphate by ferric phosphate, NaCl by KCl and trace vitamin and mineral solution by Trace Metals Solution (see Appendix 1.2).
[b] See Appendix 1.2.

Preparation:

Allow the ferric phosphate to dissolve overnight in the distilled water. Add remaining ingredients and adjust pH to 7.0. Boil for 2 min, while cooling sparge medium and headspace with O_2-free N_2 or He. Continue sparging and dispense medium into serum bottles observing strict anaerobic techniques. Autoclave to sterilize. Substrates are added as sterile anaerobic solutions just prior to inoculation.

Appendix 1.9 Medium WR86 (Fedorak and Hrudey, 1984)

Stock components used in the medium[a]:

	Compound	Amount (g/L distilled water)
Mineral Solution 1[b]	$NaCl$	50
	NH_4Cl	50
	$CaCl_2 \cdot 2H_2O$	10
	$MgCl_2 \cdot 6H_2O$	10
Mineral Solution 2	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10
	$ZnSO_4 \cdot 7H_2O$	0.1
	H_3BO_3	0.3
	$FeCl_2 \cdot 4H_2O$	1.5
	$CoCl_2 \cdot 6H_2O$	10
	$MnCl_2 \cdot 4H_2O$	0.03
	$NiCl_2 \cdot 6H_2O$	0.03
	$AlK(SO_4)_2 \cdot 12H_2O$	0.1
Phosphate Solution	KH_2PO_4	50
Resazurin Solution	Resazurin	0.1

[a] No Vitamin B Solution was added.
[b] Made in 0.01 M HCl rather than distilled water.

Final composition of Medium WR86:

Component	Amount
Mineral Solution 1	1.0 mL
Mineral Solution 2	0.1 mL
Phosphate Solution	1.0 mL
Resazurin	1.0 mL
Sodium Bicarbonate	0.57 g
Distilled Water	97.0 mL
Sulfide (2.5% $Na_2S \cdot 9H_2O$)	1.0 mL

Preparation:

Mix all ingredients except sulfide and bicarbonate. Allow to boil for 2 min. Sparge with 30% CO_2/N_2 through a gas dispersion tube, cool medium slightly and add sodium bicarbonate. Continue sparging and cooling until pH is 6.9-7.1. Dispense medium into serum bottles observing strict anaerobic techniques. Autoclave bottled medium and anaerobically prepared sodium sulfide solution separately. Add 1% (v/v) of sterilized sodium sulfide solution just prior to inoculation. Substrates can be added to the medium before the bottles are autoclaved or as sterile, anaerobic solutions after the medium has been autoclaved.

Appendix 1.10 Brain Heart Infusion (BHI) - PHA Overlay Plates

The following amounts will make enough medium for 20 petri plates.

	Components	Amounts
Bottom layer	BHI[a]	3.16 g
	Resazurin (0.1%)	5.0 mL
	Agar	9.0 g
	Distilled water	500 mL
Overlay	PHA	0.4 g
	Resazurin (0.1%)	2.0 mL
	Agar	3.6 g
	Distilled water	200 mL

[a] This is one-eighth the concentration of normal BHI (Difco laboratories, Detroit, Michigan)

Preparation:

The bottom layer is prepared by mixing components and autoclaving to sterilize. When the sterilized solution has cooled to about 45-55°C, 5 mL of sterile 0.1% (w/v) sodium thioglycolate is added as the reducing agent. Approximately 25 mL of the medium is poured into each plate and allowed to solidify. All of the components of the overlay, except for the agar, are added together and mixed to ensure the PHA granules are evenly dispersed. The agar is then added and the resulting mixture is autoclaved to sterilize. Once the sterile solution has cooled to about 45-55°C, 2 mL of sterile 0.1% (w/v) sodium thioglycolate is added as the reducing agent. Approximately 10 mL of the overlay is evenly poured over the surface of the bottom layer.

Appendix 1.11 Overlay Plates for Detection of PHA Depolymerase Activity

The following amounts will make enough medium for 20 petri plates.

	Components	Amounts
Bottom layer	Burk's Buffer[a]	500 mL
	NH_4NO_3	15 mM
	Agar	9.0 g
Overlay	Burk's Buffer[a]	200 mL
	NH_4NO_3	15 mM
	PHA[b]	0.4 g
	Agar	3.6 g

[a] Burk's Buffer is Burk's Medium (Appendix 1.1) without ferric citrate, ammonium acetate or fish peptone.

[b] Dry, powdered extracted form of PHAs used.

Preparation:

The bottom layer is prepared by mixing components and autoclaving to sterilize. When the sterilized solution has cooled to about 45-55°C, approximately 25 mL is poured into plate and allowed to solidify. All of the components of the overlay except for the agar, are added together and mixed to ensure the PHA granules are evenly dispersed. The agar is then added and the resulting mixture is autoclaved to sterilize. Once the sterile solution has cooled to about 45-55°C, 10 mL of the overlay is evenly poured over the surface of the bottom layer.

Appendix 1.12 Semidefined *Staphylococcus* Medium (Guirard and Snell, 1981).

Component	Amount
Glucose	10 g[a]
Na_2HPO_4	7.0 g
KH_2PO_4	0.5 g
K_2HPO_4	5.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
NaCl	2.0 g
Ferric citrate	1.0 mg[b]
L-Cysteine hydrochloride	0.1 g
DL-Tryptophan	50 mg
Vitamin solution	1.0 mL[c]
Casamino acids	3.0 g
Distilled water	1.0 L

[a] Glucose, if used as carbon source, was prepared as a 1 M stock and filtered sterilized. A 1% (v/v) amount was added to autoclaved medium.

[b] Ferric citrate was prepared as a 100X stock (usually 10 mg/100 mL) and filtered sterilized into a sterile brown bottle to protect from light. It was added at 10 mL/L to medium prior to autoclaving.

[c] A concentrated (100X) vitamin stock solution was prepared consisting of (per 100 mL distilled water); nicotinic acid, 100 mg; thiamine HCl, 1 mg; and biotin, 0.1 mg. The solution was filter sterilized and added at an amount of 1 mL/L to medium prior to autoclaving.

Preparation:

Ingredients were added in order and dissolved. The pH was adjusted to 7.4. The medium was autoclaved to sterilize.